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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Duff *et al.*		
APPLICATION NUMBER:	09/247,874	EXAMINER:	Schnizer, Richard A.
FILING DATE:	February 10, 1999	ART UNIT:	1635
FOR:	THERAPEUTICS AND DIAGNOSTICS BASED ON A NOVEL IL-1B MUTATION		

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. FRANCESCO S. DI GIOVINE UNDER 37 CFR 1.132

Sir:

I, Francesco S. di Giovine, born on June 4, 1956, a University Senior Lecturer at the University of Sheffield, do hereby declare that:

1. I am one of the inventors of the above-identified application entitled "Therapeutics and Diagnostics Based on a Novel IL-1B Mutation."

2. I have an M.D. degree in Medicine and Surgery from the University of Florence (Italy), Faculty of Medicine, 1982, and a PhD degree in Molecular Immunology from the University of Edinburgh (UK), 1988, Faculty of Medicine.

3. I am actively engaged in researching genetic predispositions to various inflammatory diseases.

4. Working under my direction, members of my laboratory in early 1996 discovered and sequenced the IL-1B allele having a "C" rather than a "G" at the position corresponding to +6912 in Figure 1, which is nucleotide 8845 of SEQ ID NO: 1. This allele is also referred to as IL-1B (+6912) allele 2.

5. A copy of the laboratory notebook ("the Notebook") kept by my assistant Carol Campbell and reviewed by me on an ongoing basis is attached hereto as Appendix A. This notebook contains experiments performed in my laboratory from August 24, 1995 to March 22, 1996. The page numbering is located in the lower left or right-hand corner of each sheet. I confirm that this is a true and complete copy of the notebook kept by Ms. Campbell during the time-period in question.

6. As described in the instant application (Example 1, pp. 36-37), a PCR product corresponding to the 3'UTR of the IL-1B gene was amplified from human genomic DNA and sequenced. The primers used in the amplification were disclosed in the application as SEQ ID NO: 3 (5'-GTCCCCACATTCTGATGAGCAAC-3') and SEQ ID NO: 4 (5'-TGCAGCACTCAGCAATGAGGAG-3'), which bind to regions of the IL-1B gene corresponding to positions +6720 to +6742 and +7102 to +7123, when the IL-1B gene is numbered in accordance with the numbering of Figure 1. These primers were designed on August 29, 1995 and are represented as primers F_2 and B_1 on page 3 of the Notebook. The determination that a G to C change at the +6912 location was performed by sequencing of the fragment amplified by the F_2 and B_1 PCR primers, the result of which was recorded on February 21, 1996 on page 116 of the Notebook. The oligonucleotide primer used for sequencing of the +6912 allele 2 is shown on page 117. A sequence-specific oligonucleotide primer hybridizing to nucleotides +6913 to +6947 of the IL-1B gene is described on page 118. The location of the G to C change is further evidenced on page 124, entitled "Further plans for the +8845 polymorphism," because as stated above, nucleotide 8845 of SEQ ID NO: 1 corresponds to the IL-1B (+6912) location.

7. I understand that the Examiner, while admitting that we have discovered a G to C polymorphism at position +6912, stating that neither the specification or my previously filed declaration supports a 9721 nucleotide sequence of IL-1B with a C at position +6912. (See, Office action at page 5). The Examiner states that "[s]ince polymorphisms can occur throughout a molecule, one cannot assume that there are no other polymorphisms linked to position +6912 within the 9721 bases of the IL-1B gene, and that the sequence of the rest of the 9721 nucleotides is identical to that reported in the prior art." (See, Office action at pages 5-6).

8. I believe that the C polymorphism at position +6912 is a single nucleotide polymorphism, or "SNP." A SNP is known in the art as a DNA sequence variation among individuals in which the purine or pyrimidine base (as guanine) of a single nucleotide in the genome has been replaced by another such base (as cytosine). Therefore, I believe that one of skill in the art would recognize that the sequence of the rest of the 9721 nucleotides of IL-1B is identical to the wild-type sequence, which has been reported in the art, and that upon identifying the SNP at position +6912, it was unnecessary to re-sequence the entire IL-1B gene.

9. Regarding the Examiner's statement regarding linkage analysis of the IL-1B +6912 with other IL-1B polymorphisms, I note that genetic linkage between alleles of a given gene such as IL-1B does not indicate the presence of multiple sequence variations in the IL-1B gene of an individual. Rather, I believe that linkage analysis is useful to demonstrate co-segregating polymorphisms that contribute to a given disease or disorder. As shown in the Notebook, under my direction, linkage analysis was performed on or about February 23, 1996 between the IL-1B (+6912) locus and the IL-1B *taq* locus, and the result is shown on page 129 of the Notebook. This analysis demonstrates that the IL-1B (+6912) allele 1 (termed "Allele (G)" in the notebook) is 100% associated with the IL-1B *taq* allele 2, while the IL-1B (+6912) allele 2 (termed "Allele (C)" in the notebook) is 100% associated with the IL-1B *taq* allele 1, further

demonstrating that my laboratory had identified a novel allele having a "C" at position +6912 of the IL-1B gene.

10. At the time of these experiments, the human IL-1B gene sequence published by Clark *et al.* (Nucleic Acids Research 14(20):7897-7914 (1986)) was regarded as the standard sequence for human IL-1B. This sequence is also deposited in GenBank under the accession number X04500. The Clark *et al.* sequence shows a "G" at position +6912, in contrast to the allele we discovered, which contains a "C" at position +6912. We named the "G" variation "allele 1" and the "C" variation "allele 2." The statement in the application indicating that the IL-1B allele 1 has a cytosine at position +6912 and that allele 2 has a guanine at that position is a typographical error. I believe that one of skill in the art would recognize the existence of this error based on the teachings of the application and, further, in view of the contents of the Notebook. Our measurements of allele frequency presented in the patent application (*e.g.*, Example 2, pp. 37-38) demonstrate that allele 1 is the more frequent allele and may therefore be considered the wild-type allele. Moreover, multiple publications and database entries have presented the Clark *et al.* nucleic acid sequence as the wild-type sequence. (See, *e.g.*, US Patent numbers 5,686,246; 6,720,141; 6,730,476; and 6,746,839; and GenBank Accession number P01584).

11. In conclusion, in February, 1996, prior to the time of filing of the present application, I was in possession of a novel IL-1B sequence with a "C" at position +6912 from the transcription start site, which is position 8845 of a nucleic acid sequence numbered in accordance with SEQ ID NO: 1. This allele is termed the IL-1B (+6912) allele 2 and is substantially less common than the IL-1B (+6912) allele 1 known in the prior art.

Applicants: Duff *et al.*
U.S.S.N.: 09/247,874

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Francesco di Giovine

FRANCESCO S. DI GIOVINE, M.D., Ph.D.

Dated: January 31st, 2005

Graham 2855-322

Perkin elmer. Seq

User No
13

PCR lab.

23

Mohammed - Work 0116 254 1414 bleep 4099

- Home 0116 232 2675

Franco Hotel Fax:- 03 9622 8877

10 Hrs diff - Fax @

12pm ⇒ 10am

GRANT No. [56833]

(M) PNAS - 1993 90) 6 · 2295.

(D) J o. immun 94 153-2 712.

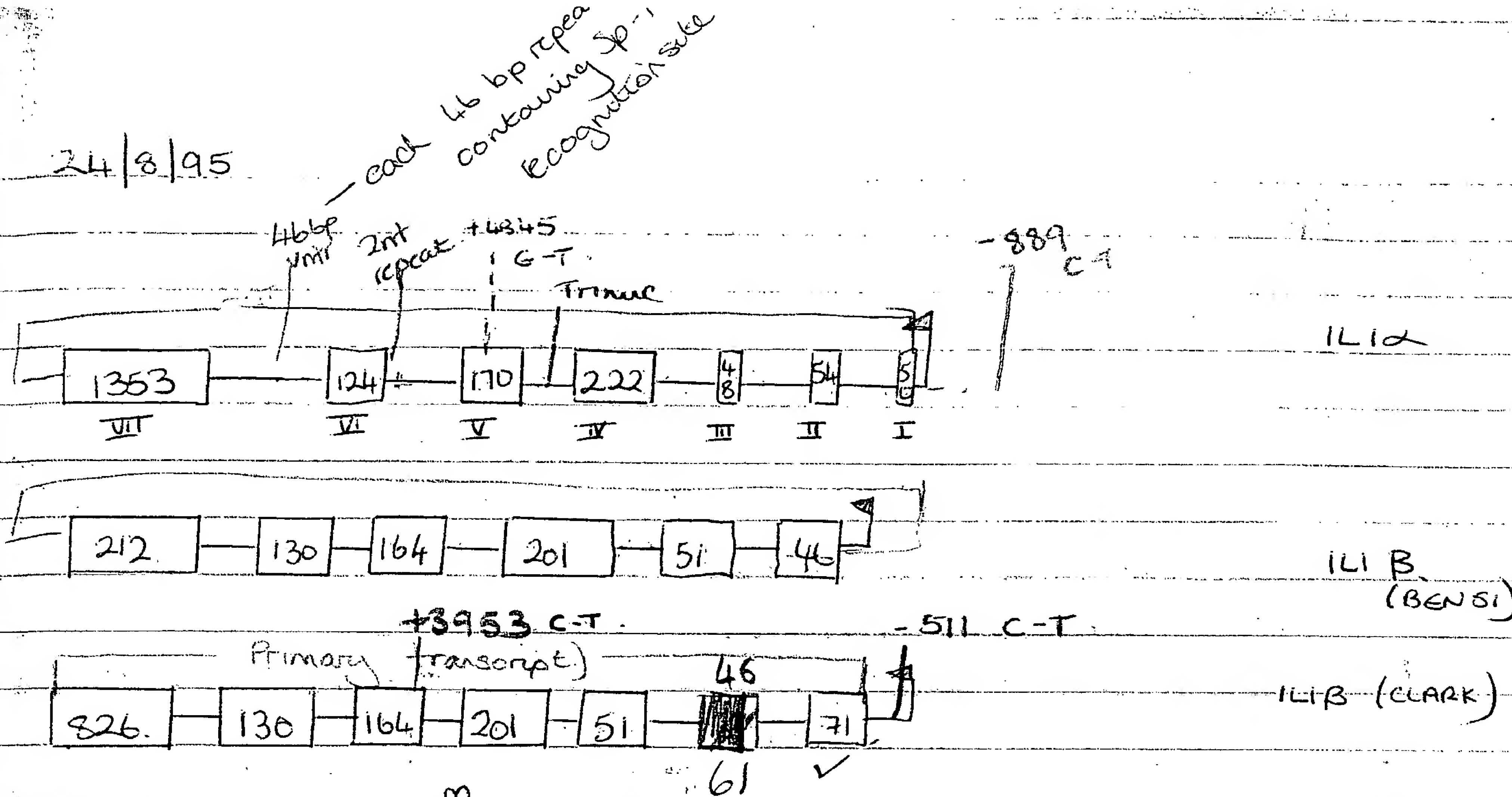
(M) ADVANCES IN NEUROIMMUNOLOGY 92 2 No 1 1 -

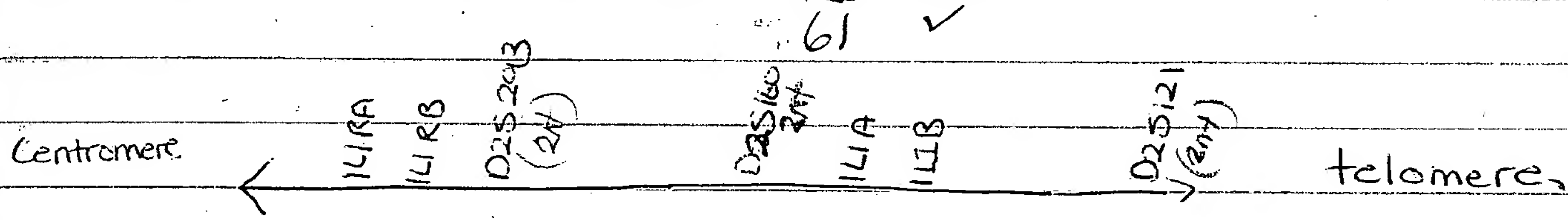
(D) Int J. immuno. pharm. 92 16 No 3

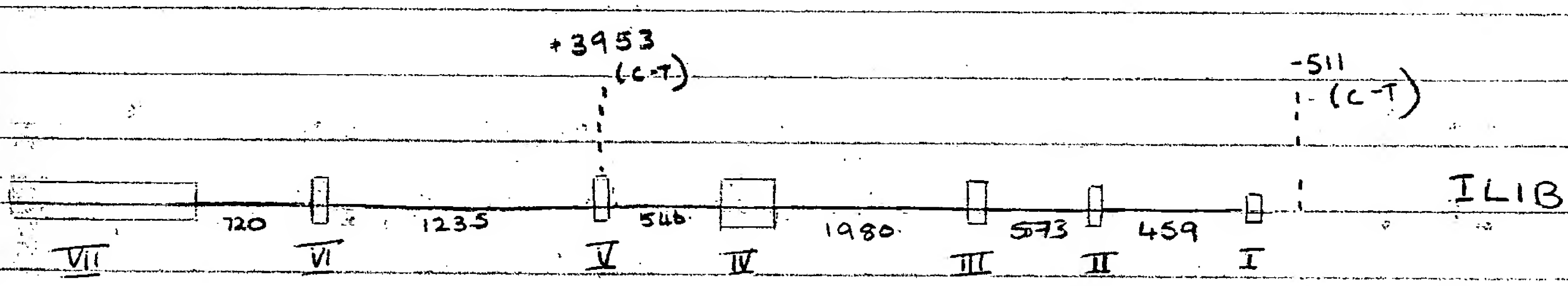
(M) MOL & CEL BIOL 95 15 1 112-119

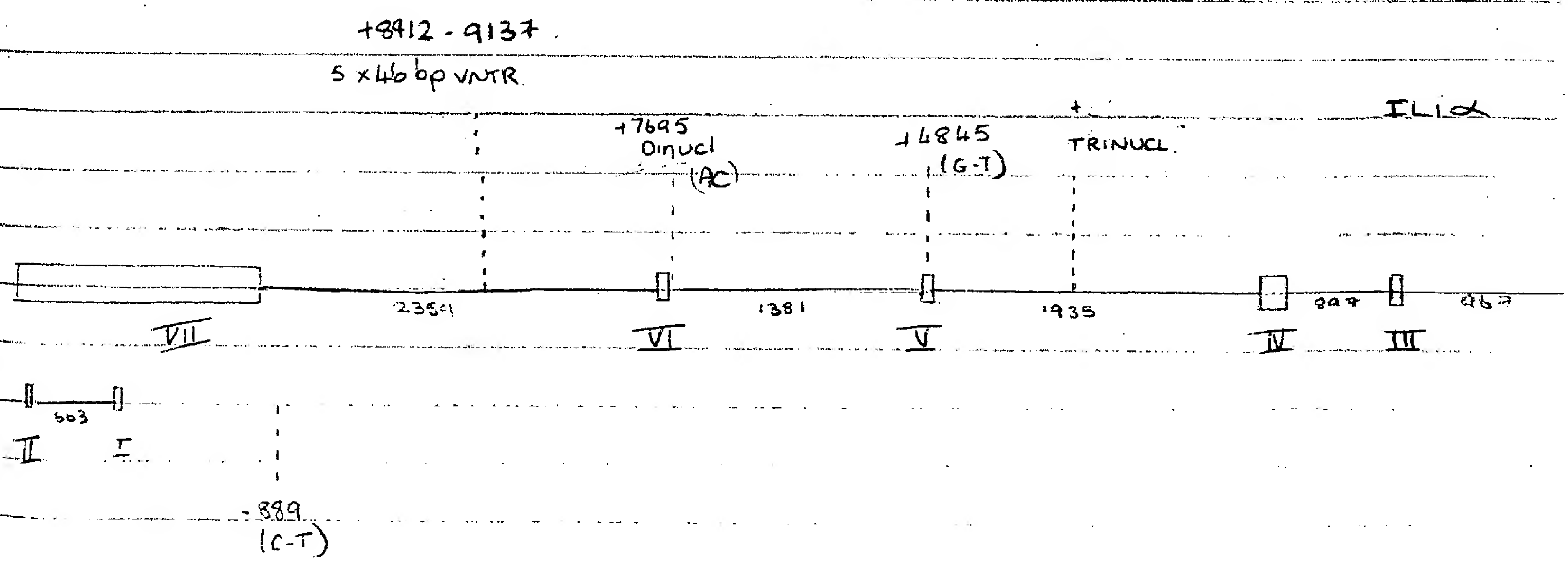
(M) MOL IMM 1995 32 NO 8 541-

24/8/95


each 46 bp repea containing Sp-1 recognition site
46bp vntr
2nt repeat
+4845 G-T
Trinuc
-889 C-T
1353
124
170
222
48
54
56
VII
VI
V
IV
III
II
I
IL1α
212
130
164
201
51
46
IL1 B (BENSI)
+3953 C-T
-511 C-T
Primary transcript
46
826
130
164
201
51
71
61
IL1β (CLARK)


Centromere
IL1RA
IL1RB
D2S2013 (2H)
D2S160
IL1A
IL1B
D2S121
telomere


+3953 (C-T)
-511 (C-T)
IL1B
720
1235
546
1980
573
459
VII
VI
V
IV
III
II
I


+8412 - 9137
5 x 46 bp VNTR
+7695 Dinucl (AC)
+4845 (G-T)
TRINUCL
IL1α
2359
1381
1935
VII
VI
V
IV
III
503
II
I
-889 (C-T)

Primer 1:- IL1B - Annealing temperature 55.9°

Primer 2:- IL1A - Anealing temperature 53.4°

IL1B

PMA~~LPS~~ stimulation - -2982 - -2795

LPS stimulation -3757 - -2729

NF κB sites (within promotor) -296/-286

-2761/-2753

29/8/95

Macrophage migration Inhibitory Factor (MIF)

- Exists preformed in macrophages & monocytes
- MIF inhibits TNF secretion
- Inhibits stimulated NO secretion by macrophages.
- 90% identity between mouse and human.
- MIF secretion is induced by TNF α, IFN Gamma.
- ∠ 1Kb - 3 exons separated by 2 introns of 189 & 95bp.
- Multiple genes in mouse but not human.

Primers - IL-1β 3'

Primers were designed using macvector. These primers span 3' AU rich region of IL1β.
The AU rich region confers instability upon an mRNA sequence - thus cytokines do ~~not~~ have them in order to return cytokine levels to norm as soon as an attack is over (TATTTAT.)

F_1 5' CAAG CAGAAAA CATGCCC GTC 3' (Tm 57.0)

F_2 5' GCTCCC ACA TTCT GATG AGCAAC 3' (Tm 57.4)

~~F_3 5' CATTCT GATGAGCAA CGGCTTC 3'~~ (Tm 56.2)

B_1 5' TGCAGCACT CAGCAA TGAGGAG. 3' (Tm 57.8)

30/8/95

Primers spanning the A-T region of IL1-α were also designed

F4 - 5' ATA GCA TAA GTT TCT TGG ACC TCA G. 3'

F5/ccc/IL1β - 5' CAG ATA CTG GAAAA CC A GGC GTAGG 3'

B2/ccc/IL1α 5' - GCT TGT AGG ACT TGA TTG CAGGTG C 3'.

F4/IL1α/ccc + B2/IL1α/ccc give a 522 bp fragment.
F5/IL1α/ccc + B2/IL1α/ccc give a 984 bp fragment

Primers were designed using 'macvector' and checked using BLAST searching to ensure that they matched no other sequence in the human genome

GIBCO BRL

Page 1 of 4

Taq DNA Polymerase

Cat. No. 18038-026

Lot No. FET401 500 units; 5 U/µl

Exp. Date: 05/97. Store at -20°C (not frost-free).

LICENSED FOR PCR

Description:
Taq DNA Polymerase is isolated from *Thermus aquaticus* YT1. The enzyme consists of a single polypeptide with a molecular weight of approximately 94 kD. *Taq* DNA polymerase is heat-stable and will synthesize DNA at elevated temperatures from single-stranded templates in the presence of a primer. Effective January 1, 1994, Life Technologies™ has modified the unit assay for *Taq* DNA polymerase, effectively doubling the enzyme concentration for consistent PCR performance.

Components:

18038-026	*Taq* DNA Polymerase	Lot No. FET401
Y02028	10X PCR Buffer	Lot No. FDJ102
Y02016	50 mM Magnesium Chloride	Lot No. FCC102
90238	1% W-1	Lot No. FET403

Unit Definition:
One unit incorporates 10 nmol of deoxyribonucleotide into acid-precipitable material in 30 minutes at 74°C.

Storage Buffer:
20 mM Tris-HCl (pH 8.0)
0.1 mM EDTA
1 mM DTT
50% (v/v) glycerol
Stabilizers

Unit Assay Conditions:
25 mM TAPS (pH 9.3)
50 mM KCl
2 mM $MgCl_2$
1 mM DTT
0.5 mg/ml activated salmon sperm DNA
0.2 mM dATP, dCTP, dGTP, dTTP

10X PCR Buffer:
200 mM Tris-HCl (pH 8.4)
500 mM KCl

The PCR Buffer supplied as a 10X concentrate should be diluted for use.

The 1% solution of the detergent W-1 can be added at a final concentration of 0.05% (v/v) and may improve the thermostability of the enzyme. Store solution at -20°C and thaw at 37°C before use.

Doc. Rev.: 022895

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4

P.C.R.

dNTP mix — 10µl dATP
10µl dCTP
10µl dGTP
10µl dTTP.
360µl H_2O ⇒ 10mM working stock.
400µl.

P.C.R buffer - supplied as 10x ⇒ working conc^n = 1x.

1µl detergent per 1µl Taq.

INITIAL DENATUR^N - 96° - 2'
DENATUR^N 94° - 1'
56°C Anneal 1'
ELONG^N 72° 1'
} X 35

FINAL ELONG^N 72° 5'
4°

D/S DNA	-	1 O.D	= 50µg/ml
RNA		1 O.D	= 40µg/ml.
S/S DNA		1 O.D	= 33µg/ml.

P.C.R. IL-1β 3' END

Reagent	Stock	use	final
10 × P.C.R. buffer	-	5μl	1 ×
$MgCl_2$	50mM	2·5μl	2·5mM
dNTP's mix	10mM (each)	4μl	0·2mM
Taq Pol (GIBCO)	5u/μl	0·2μl	1u
W-1 (detergent)	-	0·2μl	-
Template	50μg/ml	2μl	100ng/reaction
Primer mix	2μM each	5μl	1μM
H_2O	-	31·1	-
		50μl	

2 drops mineral oil.

$F_1 + B_1$ annealing Temperature = 56·0°.

$F_2 + B_1$ annealing Temperature = 53·3°.

P.C.R. CYCLES

Contents of Sto 3:

step 0: temp: 96.0 deg C time: 0h 2m 0s slope: 2.00 deg/sec time-inc: 0 sec

begin cycle 35 times:

step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec

step 2: temp: 53.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec

step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec

} × 35

end cycle 35 times

step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec

step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec

total runtime (approx.): 2h53m59s

If a P.C.R. Reaction does not work first time - alter Mg.Cl conc^n or annealing Temperature or

1/9/95

Primers F1/IL-1β/ccc
F2/IL1B/ccc
B1/IL-1β/ccc

were made and supplied in dry form. They were extracted and purified by ethanol ppt^n.

mm 737 - B1/IL1β/ccc.
mm 738 - F2/IL1B ccc
mm 739 - F1/IL1B/ccc.

	B1	F1	F2
1) Resuspend oligo in 200µl H_2O	✓	✓	✓
2) Place in eppendorf	✓	✓	✓
3) Add 10µl 3M NaAc.	✓	✓	✓
4) Add 300µl 60% etoH	✓	✓	✓
5) -70°C 1hr. - 11:30am	✓	✓	✓
6) Spin 15' 12,000 rpm	✓	✓	✓
7) Remove Supernatant	✓	✓	✓
8) Wash in 70% ETOH	✓	✓	✓
9) Remove supernatant.	✓	✓	✓
10) Dry in Vacuum ~10'	✓	✓	✓

* When you release vacuum - turn black mark away from hole - ie: off.
Remove hose & turn off H_2O first
*: Otherwise you will flood your samples

AFTER DRYING THE OLIGO SHOULD BE RESUSPENDED IN 500µl

O.D Programme 10 - Walburg. step - 10
press RS
Recall press 0 ⇒
insert blank
H_2O flush

Put tube in touch fill press RS

O.D. CALCULATION-

add 170 → 200µl. - take 5µl + 995 H_2O

A320 low.

B1 A_{260} 0.3188 = 3

= [ ] = 10.52 mg/ml

Mw = 6787.4 6787.4g/ltre = 1M

6784 mg/ml = 1M

10.52 mg/ml = 1.55mM

= 1550µM ⇒ dilute 1:77.5

1µl + 77µl H_2O

400µM soln. ⇒ 1 in 4 soln = 195µl H_2O + 65µl primer

Primer mix - 4µl B1 + 4µl F1 + ~~76~~ 152µl H_2O = 260µl stock

SAMPLE	A320	A280	A260	280/260	260/280	PROTEIN	NUCLEIC ACID
B1	1.0000	0.0191	0.2098	0.3188	0.6362	1.5718	68.957	11.988
F1	2.0000	-0.004	0.1123	0.1435	0.7883	1.2685	68.813	5.0951
F2	3.0000	-0.025	0.1237	0.1154	1.0593	0.9440	124.46	3.4762
H_2O	4.0000	-0.020	0.0000	0.0934	0.1770	5.6494	-54.74	6.4125

- 394 Synthesis Setup Listing - (Version 2.00)

Column 1 14:13:08 ,30/ 8/95

Run ID : MM 737
Cycle : 002 UMOL
End Proc: End CESS (DMT = Off)
Sequence: Seq01

Average
Step-wise
Yield : 98.4
Total bases = 22
A= 7, G= 7, C= 5, T= 3, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 6787.4

0056

5'> TGC AGC ACT CAG CAA TGA GGA G <3' B1 / IL1β / CCC

0035

T = 3

Column 2 14:13:09 ,30/ 8/95

Run ID : MM 738 G = 7
Cycle : 002 UMOL C = 5
End Proc: End CESS (DMT = Off) A = 7
Sequence: Seq02

Average
Step-wise
Yield : 96.8
Total bases = 23
A= 6, G= 4, C= 8, T= 5, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 6965.6

5'> GCT CCC ACA TTC TGA TGA GCA AC <3' F2 / IL1β / CCC

Column 3 14:13:09 ,30/ 8/95

Run ID : MM 739
Cycle : 002 UMOL
End Proc: End CESS (DMT = Off)
Sequence: Seq03

Average
Step-wise
Yield : 96.8
Total bases = 21
A= 8, G= 4, C= 7, T= 2, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 6390.2

5'> CAA GCA GAA AAC ATG CCC GTC <3' F1 / IL1β / CCC

ster (1000)

1646
3550.4
2336
3638.4
10770.8

40 μM

⇒ Stock Soln. (400 μM)

38082

1

546 μM

1.4 soln.

91 μl H_2O = 156 μl soln.

1:20 dil^n.
H_2O

F1

A260 = 0.1435 × dilⁿ factor (1000)

= 143.5 × 33

= OD of 4.735.

1446
3550.4
2336
3838.4
10770.8

Mw = 6390.

6390 g/L = 1M.

6390 mg/ml = 1M

~~6390 ng/µl = 1M.~~

4.735 mg/ml = 740 µM.

→ 1:2 ⇒ Stock Solⁿ. (400µM)

= 65µl primer + 130µl H_2O

F2 A_{260} = 0.1154 × 1000 =

115.4 × 33 = 3808.2

= 3.808 mg/ml.

Mw = 6965

6965 g/L = 1M.

6965 mg/ml = 1M.

3.808 mg/ml = 546µM

⇒ to get 400µM solⁿ 1:1.4 solⁿ

65µl primer + 91µl H_2O = 156µl solⁿ.

To get from 400 → 20 µM: 1:20 dilⁿ.

Primer mix = 2µl (each primer) + 76µl of H_2O.

P_1 = B_1 + F_1 ×4

P_2 = B_1 + F_2

IL-1B TEST PCR

Two P.C.R reactions were set up 1 with F1/IL1B/ccc+ B1/IL1B/ccc (803 bp fragment) and 1 with F2/IL4B/ccc + B1/IL-1B/ccc (403 bp fragment)

Template used = 3 x normal genomic samples

⇒ Set up 8 reactions

Master mix:- Buffer - 40µl ✓
$MgCl_2$ - 20µl ✓
dNTP's - 32µl ✓
Taq Pol - 1.6µl ✓
W1 - 1.6µl ✓
Template
H_2O 248µl ✓
344.0µl

Pipette 43µl of Master mix into each tube ✓
into tubes 1,2,3 and 4 add 5µl Primer mix 1 ✓
into tubes 5,6,7 and 8 add 5µl Primer mix 2
tubes 1 and 8 are H_2O controls

TUBE NO.			* DNA USED German controls
1/	- H_2O control	+ Primer mix 1	H_2O
2/		+ Primer mix 1	Sample No 1
3/		" "	" " 3
4/		" "	" " 7
5/		+ Primer mix 2	" " 8 (oops Primer 1 added by mistake)
6/		" "	1
7/		" "	3
8/	- H_2O control	+ Primer mix 2	H_2O

Tubes are 'loaded' as above - before adding DNA - Wax pellets are added to each tube they are then melted @ 75°C for 5' and cooled to 4°C for 3' DNA samples (2µl) are then added on top of the wax layer before putting on the thermocycler.

No 61 - 75° 5' } wax melting
62 4° 3' }

* The volume of reagent which should be put on top of the wax layer should be 10-30µl - To ensure equal mixing this can be achieved by adding Master mix to a DNA / H_2O solution which is wax sealed *

IL-1α Primers DATA

- 394 Synthesis Setup Listing - (Version 2.00)

Column 1 13:50:56 ,31/ 8/95

Run ID : MM 740
Cycle : 002 UMOL
End Proc: End CESS (DMT = Off)
Sequence: Seq01

Average
Step-wise
Yield : 98.5
Total bases = 25
A= 4, 6= 10, C= 3, T= 8, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 7775.0 (4451)

5'> GCT TGT AGG ACT TGA TTG CAG GTG G <3' (11420) B2/CCC/IL1α

Column 2 13:50:57 ,31/ 8/95

Run ID : MM 741
Cycle : 002 UMOL
End Proc: End CESS (DMT = Off)
Sequence: Seq02

Average
Step-wise
Yield : 98.8
Total bases = 25
A= 7, 6= 6, C= 5, T= 7, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 7676.0 (11426)

5'> ATA GCA TAA GTT TCT GGG ACC TCA G <3' F4/CCC/IL-1α

Column 3 13:50:57 ,31/ 8/95

Run ID : MM 742
Cycle : 002 UMOL
End Proc: End CESS (DMT = Off)
Sequence: Seq03

Average
Step-wise
Yield : 98.2
Total bases = 25
A= 9, 6= 8, C= 5, T= 3, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 7742.0

5'> CAG ATA CTG GAA AAC CAG GCG TAG G <3' F5/CCC/IL1B

5' GCT TGT AGG ACT TGA TGG
AGG TGG 3'

3'
TTA GCA TAA GTT TCT GGG ACC TCA
3'
ATA CTG GAA AAC CAG GCG TAG

52.7° ⇒ 522bp fragment
54.6° ⇒ 984bp fragment

1174 5'
1429
1437

IL-12 Primers DATA

- 394 Auto Analysis Listing -

Time: 16:18:39 , 1/ 9/95

Column £1		Column £2		Column £3	
Seq: Seq01 B2		Seq: Seq02 F4		Seq: Seq03 F5	
Overall: 69.9		Overall: 75.1		Overall: 66.0	
ASWY: 98.5		ASWY: 98.8		ASWY: 98.3	
Num Base	ASWY	Num Base	ASWY	Num Base	ASWY
2 G	100.0	2 A	100.0	2 G	100.0
3 T	97.1	3 C	92.5	3 A	97.3
4 G	94.9	4 T	94.9	4 T	97.7
5 G	96.2	5 C	96.2	5 G	97.6
6 A	96.9	6 C	96.9	6 C	96.2
7 C	95.9	7 A	97.4	7 G	96.8
8 G	96.4	8 G	97.8	8 G	96.7
9 T	96.9	9 G	98.1	9 A	96.6
10 T	97.2	10 G	98.3	10 C	96.9
11 A	96.8	11 T	98.5	11 C	97.2
12 G	97.1	12 C	98.6	12 A	97.5
13 T	97.4	13 T	98.7	13 A	97.7
14 T	97.6	14 T	98.8	14 A	96.8
15 C	97.6	15 T	98.9	15 A	97.1
16 A	97.8	16 G	99.0	16 G	97.3
17 G	97.9	17 A	99.0	17 G	97.4
18 G	98.1	18 A	99.1	18 T	97.6
19 A	98.0	19 T	98.8	19 C	97.7
20 T	98.1	20 A	98.9	20 A	97.8
21 G	98.2	21 C	98.6	21 T	97.9
22 T	98.3	22 G	98.6	22 A	98.0
23 T	98.4	23 A	98.7	23 G	98.1
24 C	98.5	24 T	98.8	24 A	98.2
25 G	98.5	25 A	98.8	25 C	98.3

B2|ccc|IL12 = 5' GCT TGT AGG ACT TGA TGG AGG TGG 3'

F4|ccc|IL12 = 5' ATA GCA TAA GTT TCT GGG ACC TG 3'

F5|ccc|IL12 = 5' CAG ATA CTG GAA AAC CAG GCG TAG 3'

$F_4 + B_2$ – Annealing Temperature = 52·7° ⇒ 522 bp fragment

$F_5 + B_2$ – Annealing Temperature = 54·6° ⇒ 984 bp fragment

4/9/95

Purify, by ethanol precipitation newly made primers B2/ILI2/CCC, F4/ILI2/CCC, F5/ILI2/CCC

1) Resuspend oligos in 200µl Pure H_2O.
2) Put 100µl into ~~FUEE~~ fresh eppendorf
3) Add 10µl 3M NaAc
4) Add 300µl EtOH (100%)
5) Freeze @ -70°C ~1hr.
6) Spin, 12000 rpm - 15'
7) Remove S/N
8) Wash pellet in 70% EtOH
9) Spin 5' 12,000 rpm
10) Dry Under vacuum ~10'.
11) Resuspend 500µl H_2O
12) Measure O.D. (Prog 10. Warburg)

* To put vacuum on - turn black nork away from 'hole' put on tube and turn on top - to turn off, pull tube off - then switch off H_2O then lift nozzle *.

B2 A_{260} - 0.5413 x dil^n factor (200).
= 108.26 X 33
= 00. = 3572.58.

MW = 7775 = 7775g/l = 1M
7775µg/µl = 1M
3572.58 = 460µM

Dilution factor = 22

SAMPLE	A320	A280	A260	280/260	260/280	PROTEIN	NUCLEIC ACI
1.0000	-0.010	0.0104	0.0161	0.7798	1.2824	11.850	0.9111
B2 2.0000	0.0630	0.3096	0.5413	0.5156	1.9397	20.491	21.209
F4 3.0000	-0.023	0.1182	0.2347	0.5475	1.8264	23.812	11.122
F5 4.0000	0.0264	0.1240	0.2105	0.5304	1.8853	12.133	8.0636

Run P.C.R. Samples (P10/11) on 1% Agarose Gel. Add 1µl EtBr to Ge
Make small 30ml gel. 0.5g Agarose
50ml TBE (1x)

- Take 10µl PCR product
- Add 4µl loading dye

also run 0.5-1µl ΦX HaeIII marker

GEL I loading order.

1) ΦX HaeIII ~~100~~ Molecular weight marker
2) H_2O control Sample No 1 (Primers B_1/F_1)
3) " 3
4) " 7
5) " 8.
6)

Gel 2

1) ΦX HaeIII Molecular Wt marker
2) ~~No 1 H_2O~~ Sample No 1 (Primers B_1/F_2)
3) No 3
4) H_2O control

Gel was run @ 80V for ~ 30'.

RESULT No bands were seen except those of primers - so re-plan expt. optimise conditions such as magnesium concentration and annealing Temperature. Increasing magnesium concentration lowers the specificity of the reaction as does lowering the annealing temp.

ILIB PCR - MARK II

OPTIMISATION

alter - $MgCl_2$

- Annealing Temp.

$MgCl_2$ - was 2.5 use:- 2.0 3.0 3.5

Annealing Temperature was 53 :- - try 50 47

56 :- 53 50

Block 1 Annealing temp: 56 °C

Samples 1, 3

Magnesium 2.0 3.0 3.5 µl

Master mix :- 1 x9		Mix 2		Mix 3	
Buffer	5µl		5µl		5µl ✓
$MgCl_2$	2.5µl	(2.5mM) ✓	3.5µl	(3.5mM)	4.5µl (4.5mM)
dNTP's	4µl ✓		4µl		4µl
Taq	0.2µl ✗		0.2µl		0.2µl
W1	0.2µl ✓		0.2µl		0.2µl
Template	2µl		2µl		2µl
Primer	5µl ✓		5µl		5µl
H_2O	31.5		~~30.5~~		~~30~~ 29 .5
	50		50		50

31.5 x9 = 283 / 4.5

30.5 x9 = 274.5

29.5 x9 = 265.5 / 4.5

	(1) mm1	(2) mm2	(3) mm3
(A) Block 1:- 56°	(c1a).(100) 1 2 H_2O	1,2 H_2O	1, 2, H_2O
(B) Block 2:- 53°	1, 2, H_2O	1, 2, H_2O	1, 2, H_2O
(C) Block 3:- 50°	1, 2 H_2O	1, 2, H_2O	1, 2, H_2O

O.D Primers

F4, A_{260} = 0·2347.

0·2347 × 200 × 33 = OD Dilution factor = 10

O.D = 1549·02

M_w = 7676·0 = IM = 7678 μg/μl

⇒ 1549 = 202 mM

F5 A_{260} = 0·2105 × 200 × 33 = OD

O.D = 1389.3

M_w = 6390

= 220 mM

F5 - dilution factor = 11 - to give 20 mM soln.

$$\frac{\left(A_{260} \times \text{dil}^n \times 0{\cdot}033 / mw\right) \times 10^6}{20} = \text{dil}^n \text{ factor}$$

F5 $\frac{(1{\cdot}3893) \times 10^6}{20} = 10$

Contents of Sto 1:
step 0: temp: 96.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: 56.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h48m11s

Samples A

Contents of Sto 2:
step 0: temp: 96.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: 53.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h52m59s

Samples B

Contents of Sto 3:
step 0: temp: 96.0 deg C time: 0h 2m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: 50.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h59m23s

Samples C

IL-1α P.C.R

PROVISIONAL.

IL 1a PCR PROTOCOL

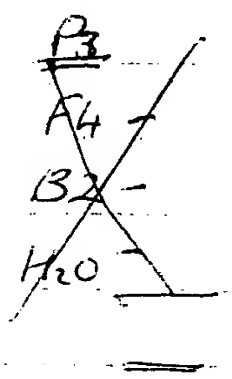
REAGENT	STOCK	USE	FINAL
10 X BUFFER		5µl	1X
$MgCl_2$	50mM	2.5µl	2.5mM
dNTP's	10mM	4µl	0.2 mM
Taq Pol	5U/µl	0.2µl	1U
W-1		0.2µl	
TEMPLATE	50µg/ml	2µl	100ng/react
primer mix	20µM each	5µl	1µM
H_2O		31.1µl	
		50µl	

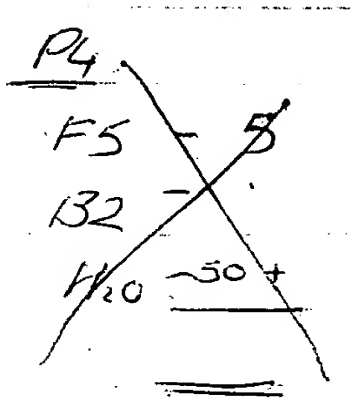
CYCLES:

INITIAL DENATURATION	96°C	2MINS	
DENATURATION	94 °C	1 MIN	
ANNEALING	F4 (52 °) F5(54 °)	1 MIN	X35
ELONGATION	72°C	1 MIN	
ELONGATION	72°C	5 MINS	
	4°C	INFINITY	

Primer mix 3 = F4/IL1α/CCC + B2/IL1α/CCC @ 20mM

Primer mix 4 = F5/IL1α/CCC + B2/IL1α/CCC @ 20mM





5/9/95

Run P.C.R samples on a 1% Agarose Gel

10µl P.C.R products
4µl bromophenol blue
load 14µl

Loading order

1)	1µl ΦX HaeIII Mw marker	18)	1µl ΦX HaeIII
2)	14µl $A_1(1)$	19)	14µl $B_3(1)$
3)	" $A_1(2)$	20)	" $B_3(2)$
4)	$A_1 H_2O$	21)	$B_3(H_2O)$
5)	$A_2(1)$	22)	" $C_1(1)$
6)	$A_2(2)$	23)	" $C_1(2)$
7)	$A_2(H_2O)$	24)	" $C_1(3)$
8)	$A_3(1)$	25)	" $C_2(1)$
9)	$A_3(2)$	26)	" $C_2(2)$
10)	$A_3(H_2O)$	27)	" $C_2(H_2O)$
11)	$B_1(1)$	28)	" $C_3(1)$
12)	$B_1(2)$	29)	" $C_3(2)$
13)	$B_1(H_2O)$	30)	" $C_3(H_2O)$
14)	$B_2(1)$	31)	" ΦX HaeIII
15)	$B_2(2)$		
16)	$B_2(H_2O)$		
17)	1µl ΦX HaeIII Mw marker		

Run gel at 120V. for ~ ½ hour

Set up 1 & 2 PCR as per provisional protocol P19.

MM(1)		MM(2)	
buffer	15µl	buffer	15µl
$MgCl_2$	7·5µl	$MgCl_2$	7·5µl
dNTP's	12µl	dNTPs	12µl
Taq Pol	0·6µl	Taq	0·6µl
W-1	0·6µl	W1	0·6µl
Primer mix (F4/B2)	6µl	Primer mix (F5/B2)	6µl
H_2O	93·3µl	H_2O	93·3
	150µl		150µl

For each primer pair use two different DNA samples and 1 water control.

FURTHER OPTIMISATION OF IL1B PCR

$MgCl_2$	- 1.0µl	1.5µl	~~5~~ 5.0µl
Temp.	57°C	60°C	63°C

	MM1		MM2		MM3
Buffer -	5 ✓	(45)	5µl	(45)	5 µl
$MgCl_2$ -	~~2.5~~ 1.0 ✓	(9)	1.5µl	(13.5)	2.0 µl
dNTPs -	4 ✓	(36)	4 µl	(36)	4 µl
Taq -	0.2	(1.8)	0.2µl	(1.8)	0.2 µl
W1 -	0.2	(1.8)	0.2 µl	(1.8)	0.2 µl
Primer -	5	(45)	5 µl	(45)	5 µl
H_2O -	32.6	~~(23.4)~~ (293.4)	32.1 µl	(288.9)	~~28~~ 31.6µl (284.4)
	48µl		48.		48µl

Contents of Sto 1:
step 0: temp: 96.0 deg C time: 0h 2m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: [illegible] slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h47m59s

Contents of Sto 2:
step 0: temp: 96.0 deg C time: 0h 2m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: [illegible] slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec

Contents of Sto 3:
step 0: temp: 96.0 deg C time: 0h 2m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: [illegible] slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h38m59s

Loading order

2)	A_1 (1)	Temp 57°C	mm 1	DNA sample 96
3)	A_1 (2)	"	mm1	" 97
4)	A_1 (H_2O)	"	mm 1	H_2O control
5)	A_2 (1)	"	mm 2	" 96
6)	A_2 (H_2O)	"	↓	97
7)	A_2 (H_2O)	"	↓	H_2O control
8)	A_3 (1)	"	mm 3	96
9)	A_3 (2)	"	↓	97
10)	A_3 (H_2O)	"	↓	H_2O control

#

11)	B_1 (1)	Temp 60°	mm 1	# 96
12)	B_1 (2)	"	↓	# 97
13)	B_1 (H_2O)	"	↓	H_2O control
14)	B_2 (1)	"	mm 2	# 96
15)	B_2 (2)	"	↓	# 97
16)	B_2 (H_2O)	"	↓	H_2O control
17)	B_3 (1)	"	mm 3	# 96
18)	B_3 (2)	"	↓	# 97
19)	B_3 (H_2O)	"	↓	H_2O control

20)	C_1 (1)	Temp 63°	mm 1	# 96
21)	C_1 (2)	"	↓	# 97
22)	C_1 (H_2O)	"	↓	H_2O control
23)	C_2 (1)	"	mm 2	# 96
24	C_2 (2)	"	↓	# 97
25	C_2 (H_2O)	"	↓	H_2O control
26	C_3 (1)	"	mm 3	# 96
27.	C_3 (2)	"	↓	# 97
	C_3 (H_2O)	"	↓	H_2O control

* ALL PCR REACTIONS ARE BEING CARRIED OUT IN 25µl TO MINIMISE WASTAGE OF REAGENTS *

To ensure that all my reagents are working and I am not doing anything silly I will try out an already proven P.C.R. - (Alisons)

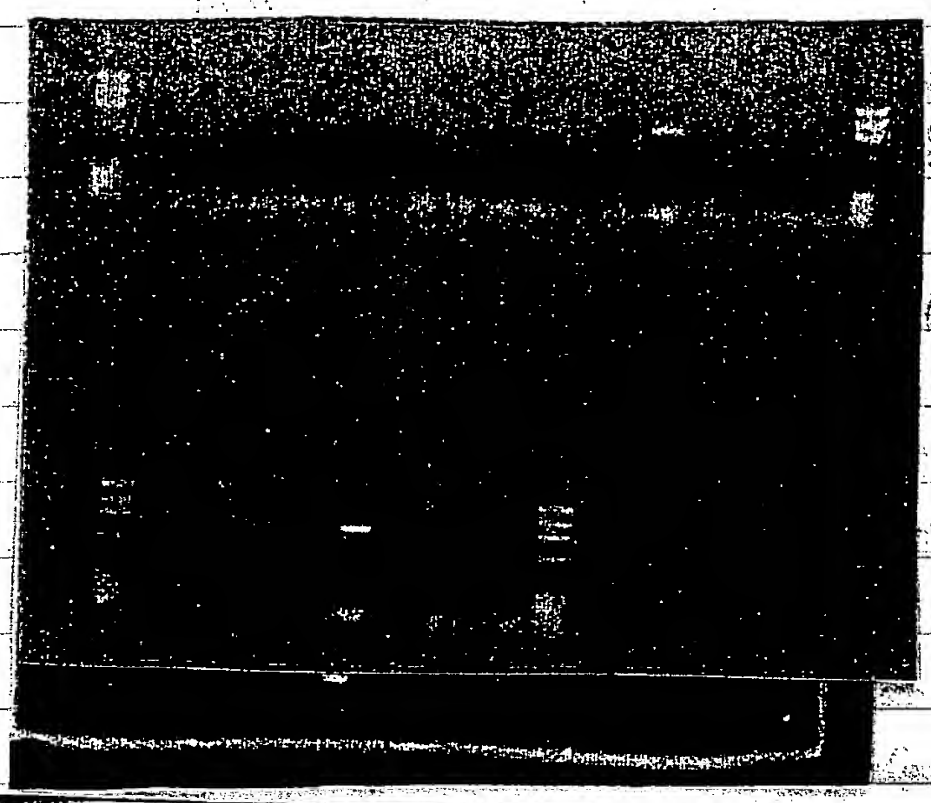
Date 5/9/95.

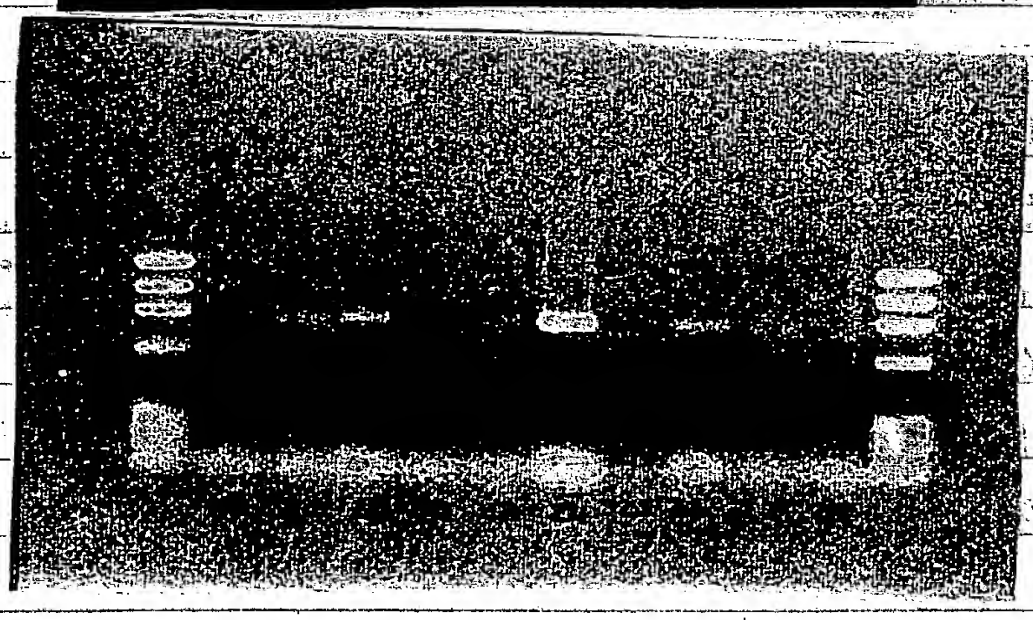
Number of Samples ....3.....

Disease...Any!.....

P'morph....Taq 1.....

Water	48.8µl
Buffer	...8....µl
Magnesium	.....8.....µl
dNTPs	.....6.4.µl
Primers	.....8.....µl
Taq	.....0.4.µl
W-1	.....0.4.µl
Template	...2...µl





11.1 B RESULTS Gel loading order P23 -

A number of bands, of the correct size were seen. Those most prominent were:

\- 60°C Annealing - 1.5µl $MgCl^{2+}$

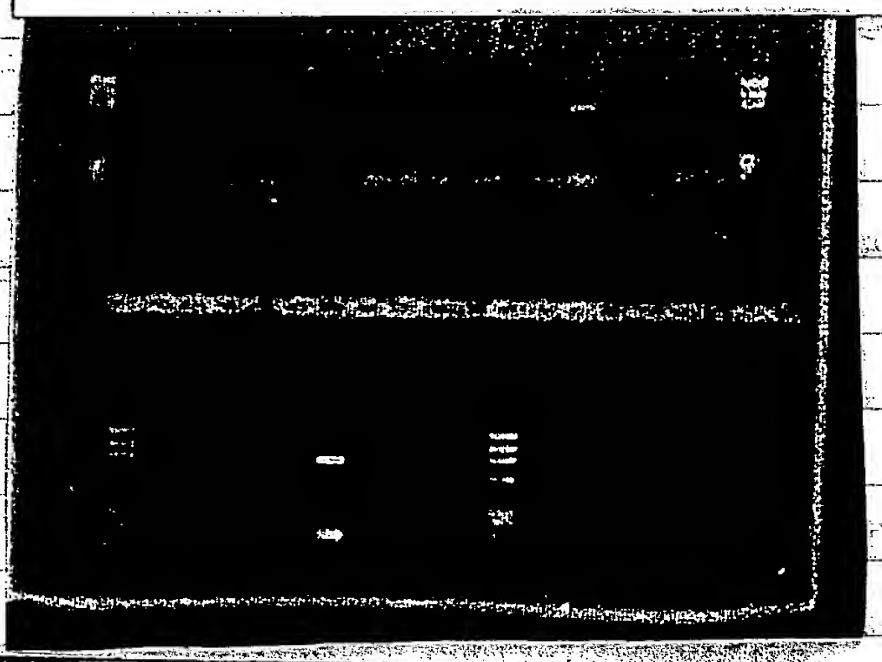
* - 63°C Annealing - 1.5µl $MgCl^{2+}$

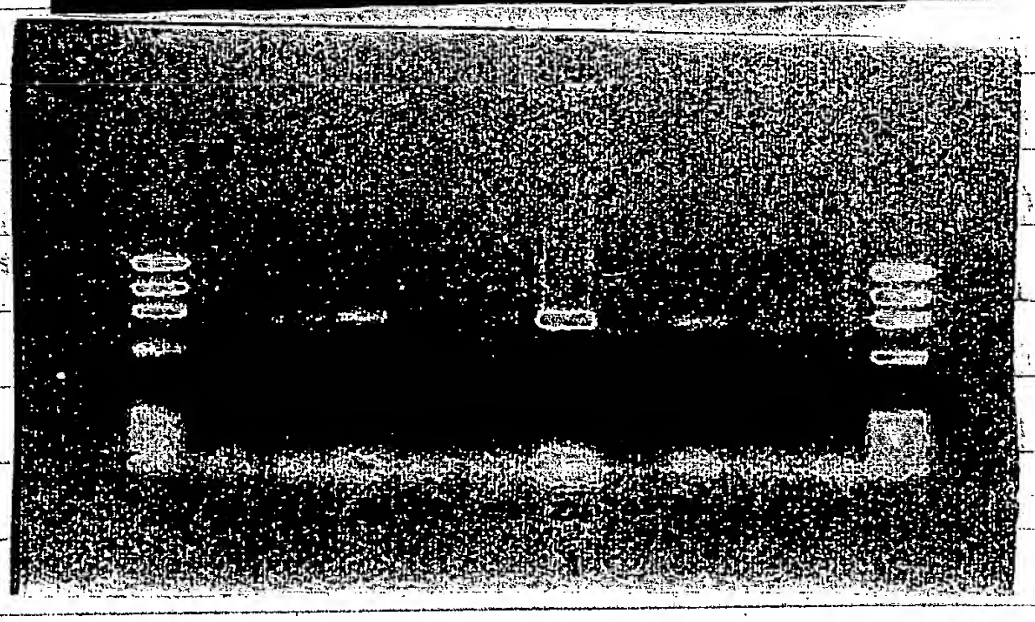
For Gel pictures see Above

To ensure that
I am not doing
already proven

Buffe
Mg
dNT
Tac
W1
Prim
H_2c

Date	5./9./95.
Number of Samples	3
Disease	Any!
P'morph	Taq 1
Water	48.8µl
Buffer	8 µl
Magnesium	8 µl
dNTPs	6.4 µl
Primers	8 µl
Taq	0.4 µl
W-1	0.4 µl
Template	2 µl





11.1 B RESULTS Gel loading order P2B -

A number of bands, of the correct size were seen.
Those most prominent were:

\- 60°C Annealing - 1.5µl $MgCl^{2+}$

* - 63°C Annealing - 1.5µl $MgCl^{2+}$

For Gel pictures see Above

OPTIMISATION OF IL12 PCR

Primers F4 / B2

	mm 1	mm 2	mm 3	mm 4	mm 5	mm 6
Buffer	~~2.5~~	2.5	2.5	2.5	2.5	2.5 (22.5)
Mg	0.5 (4.5)	1 (9)	1.5 (13.5)	2 (18)	2.5 (22.5)	3 (27)
dNTP's	~~#2~~	2	2	2	2	2 (18)
Primers	2.5	2.5	2.5	2.5	2.5	2.5 (22.5)
Taq	0.1µl	0.1	0.1	0.1	0.1	0.1 (0.9)
W-1	0.1	0.1	0.1	0.1	0.1	0.1 (0.9)
H_2O	17.3 (155.7)	16.8 (151.2)	16.3 (146.7)	15.8 (142.2)	15.3 (137.7)	14.8 (133.2)
	25	25	25	25	25	25

Predicted annealing Temp:- 53°C

DNA (Psoriasis) sample: x6 + H_2O

x6 + H_2O

H_2O

Make 3x amount - for studies at diff Temperatures
ie a total of 9x above

~~buffer~~

Carry out above PCR at same Mg^{2+} conc^n but at 50 and 56°C

50°C set: A_{1-6} + A H_2O } labelled 50°C on side
B_{1-6} + B H_2O }

56° set: A_{1-6} + A H_2O } labelled 56°C on side
B_{1-6} + B H_2O }

Summary of ILI-2 OPTIMISATION

6 different master-mixes @ 6 diff Mg conc^{n}:

1) 0.5μl
2) 1 μl
3) 1.5μl
4) 2.0μl
5) 2.5μl
6) 3.0μl

⇒ Each master mix was tested out with 2 DNA samples and water controls

⇒ Reactions were carried out at 50 - 53 and 56 °C

Loading order. IL-12 OPTIMISATION.

1)	Mw Marker	Q		
2)	DNA sample	125	mm 1	50°C
3)	"	135	1	"
4)		125	2	"
5)		135	2	"
6)		125	3	"
7)		135	3	"
8)		125	4	"
9)		135	4	"
10)		125	5	"
11)		135	5	"
12)		125	6	"
13)		135	6	"
14)		H_2O control	3	"
15)		H_2O control.	3	"

1)	Mw Marker.			
2)	Sample	125	mm 1	53°C.
3)		135	1	"
4)		125	2	"
5)		135	2	"
6)		125	3	"
7)		135	3	"
8)		125	4 - 200	"
9)		135	4	"
10)		125	5	"
11)		135	5	"
12)		125	6	"
13)		135	6	"
14)		125	3	"
15)		135	6.	"

1) Molecular wt marker

2) DNA sample 125 mm 1 56°C

3) " 135 " 1 "

4) " 125 " 2 "

5) " 135 " 2 "

6) " 125 " 3 "

7) " 135 " 3 "

8) " 125 " 4 "

9) " 135 " 4 "

10) " 125 " 5 "

11) " 135 " 5 "

12) " 125 " 6 "

13) " 135 " 6 "

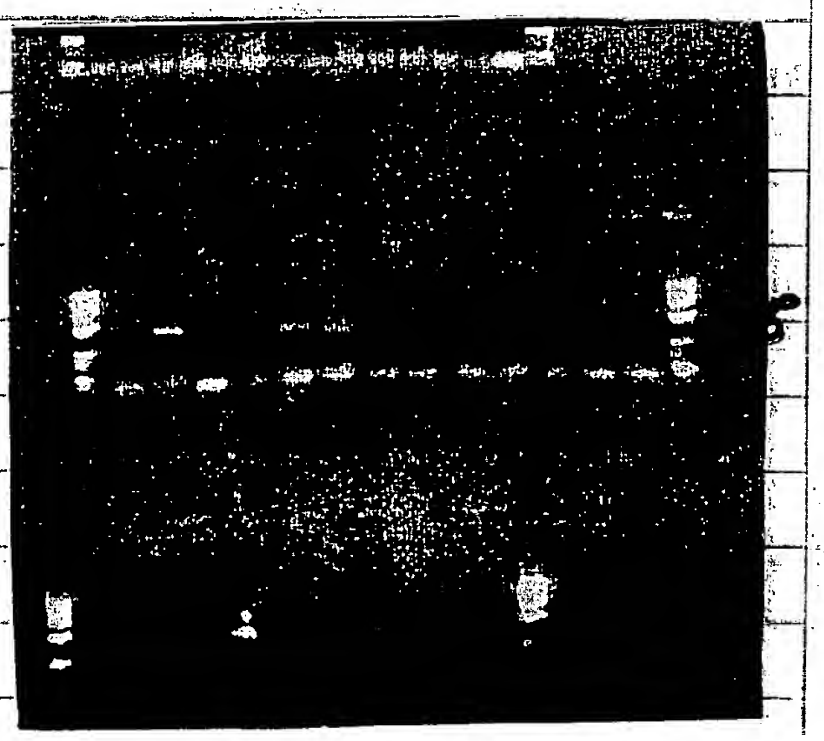
14) " 125

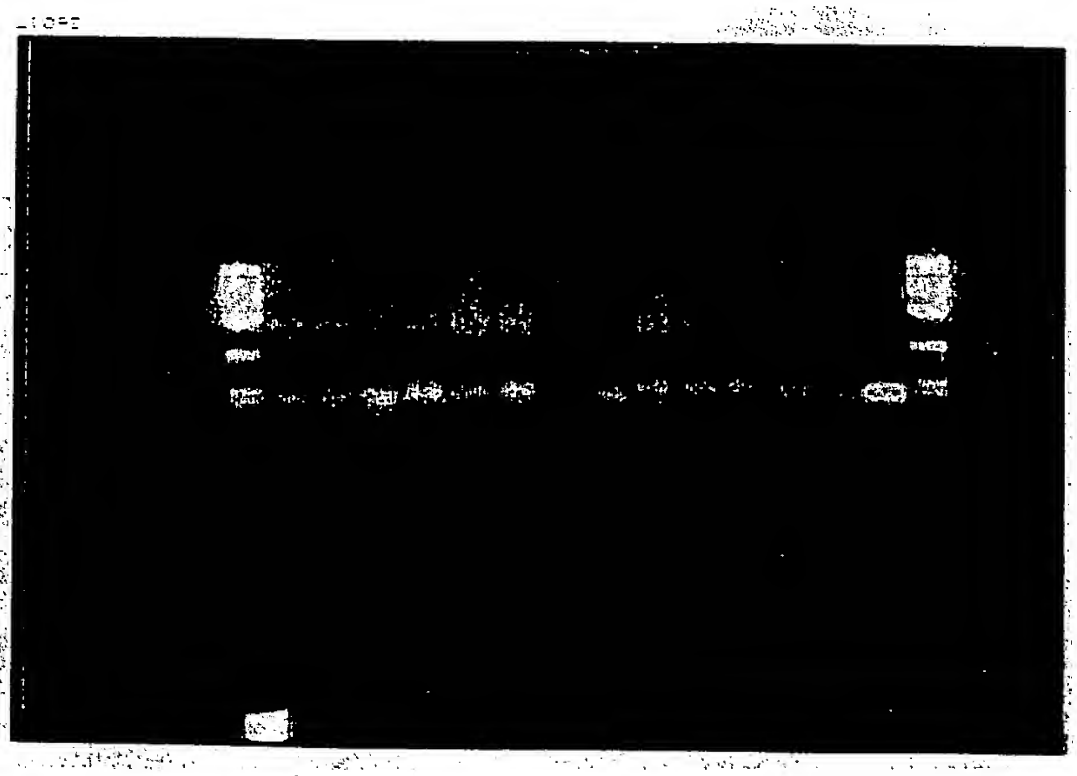
16) " 135

14) H_2O control " 6 "

15) H_2O control " 3 "

RESULTS





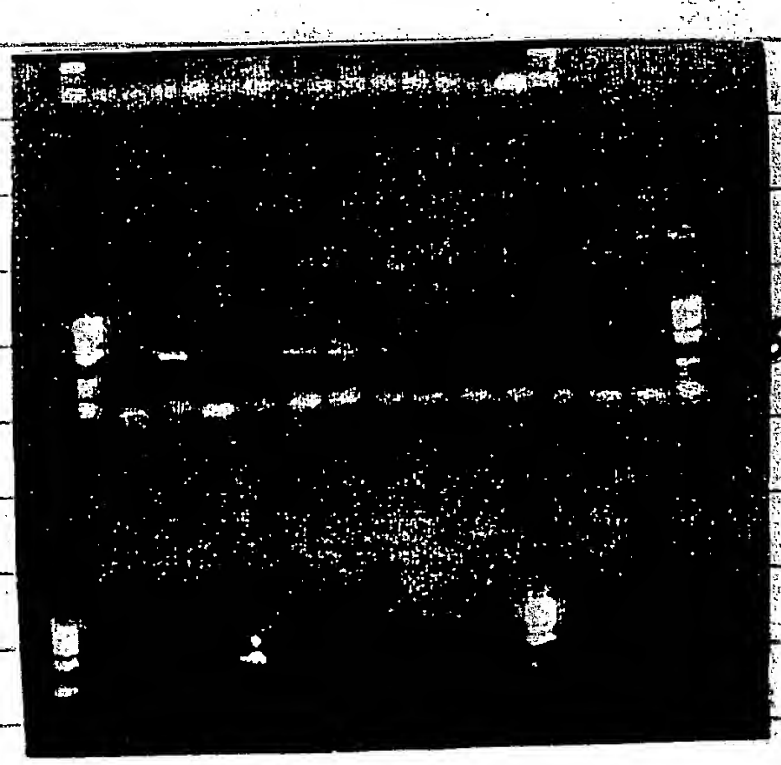
at 50°C only smears were observed

Conditions for ILI2 PCR are high temp low magnesium 53°C 1mM magnesium

1) Molecular wt marker

2)	DNA sample	125	mm 1	56°C
3)	"	135	" 1	"
4)	"	125	" 2	"
5)	"	135	" 2	"
6)	"	125	" 3	"
7)	"	135	" 3	"
8)	"	125	" 4	"
9)	"	135	" 4	"
10)	"	125		
11)	"	13		
12)	"	12		
13)	"	13		
14)	"	12		
15)	"	13		
16)	"	H_2O control		
17)	"	H_2O control		

RESULTS



at 50°C only smears were observed

From Above Gel it seems that best conditions for ILI-2 PCR are high Temp low magnesium

53°C 1mM magnesium

6/9/95

OPTIMISATION OF IL1B F2/B1 PRIMERS

Tried already:- 53°C annealing Temp.
2.5mM Magnesium.

Try now Mg 1 2 3 4 5 6 mM
Temp 50° 56° 60°

Reagent	1	2	3	4	5	6
Buffer	2.5	2.5	2.5	2.5	2.5	2.5 (22.5)
Mg	0.5	1	1.5	2	2.5	3 (27)
dNTPs	2	2	2	2	2	2 (18)
Primers	2.5	2.5	2.5	2.5	2.5	2.5 (22.5)
Taq	0.1	0.1	0.1	0.1	0.1	0.1 (0.9)
W1	0.1	0.1	0.1	0.1	0.1	0.1 (0.9)
H_2O	16.3 (146.7)	15.8 (142.2)	15.3 (137.4)	14.8 (133.2)	14.3 (128.7)	13.8 (124.2)
	24	24	24	24	24	

label samples:-

DNA sample - 157 = x
- 167 = y

IL-1β OPTIMISATION: Primers F2/B1

Contents of Sto 1:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 2: temp: 50.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 3h 2m23s

Contents of Sto 2:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 2: temp: 56.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h52m11s

Contents of Sto 3:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 2: temp: 60.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h46m11s

Loading Order

	DNA sample	Master mix	Temperature
①	Mw marker		
②	157	mm 1	50°
③	167	1	50°
④	157	2	"
⑤	167	2	"
⑥	157	3	"
⑦	167	3	"
⑧	157	4	"
⑨	167	4	"
⑩	157	5	"
⑪	167	5	"
⑫	157	6	"
⑬	167	6	"
⑭	~~157~~ H_2O		"
⑮	~~167~~. H_2O		"

①	~~157~~		
②	157	mm 1	56°
③	167	1	"
④	157	2	"
⑤	167	2	"
⑥	157	3	"
⑦	167	3	"
⑧	157	4	"
⑨	167	4	"
⑩	157	5	"
⑪	167	5	"
⑫	157	6	"

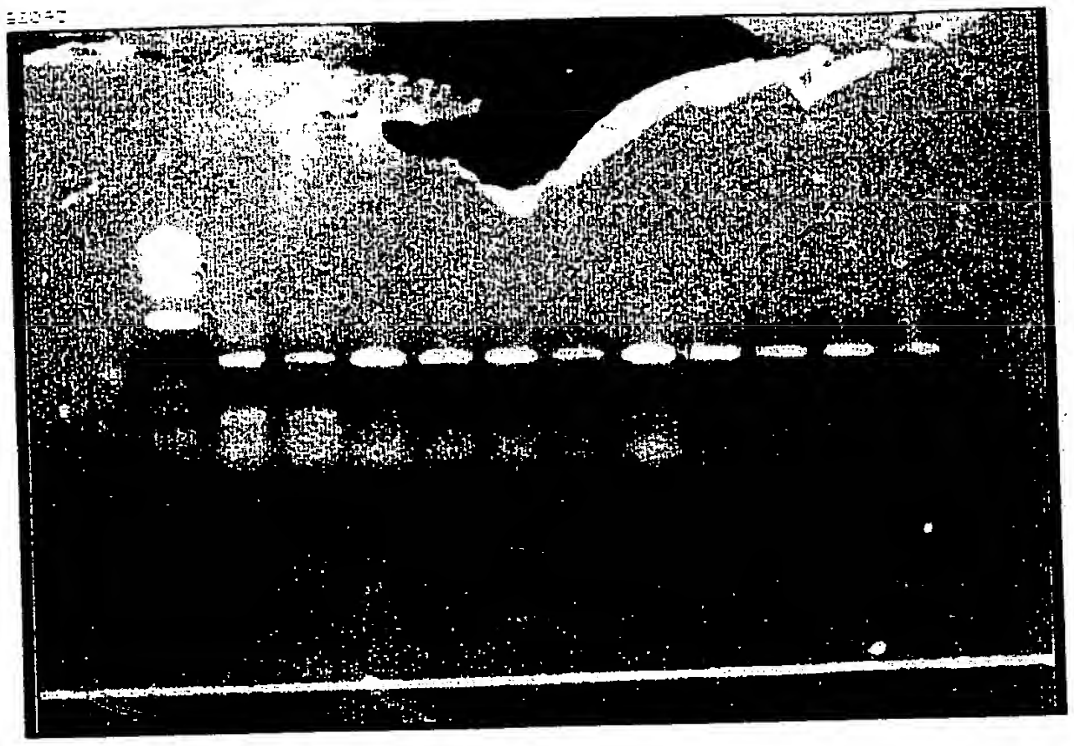
	DNA sample	Master Mix	Temperature
(13)	167	6	56°
(14)	~~157~~ H_2O	6	"
(15)	H_2O.	3	"
(16)			

(1)			
(2)	157 (X)	mm	60°
(3)	167 (X)	1	60°
(4)	157	2	60°
(5)	167	2	60°
(6)	157	3	60°
(7)	167	3	60°
(8)	157	4	60°
(9)	167	4	60°
(10)	157	5	60°
(11)	167	5	60°
(12)	156	6	60°
(13)	167	6	60°
(14)	H_2O	6	60°
(15)	H_2O	3	60°
(16)			

- 1st gel didn't run properly - Will try to Re-run with remaining samples. - 56° low Mg looked Good.
⇒ Predicted Annealing Temp = 53°.
Re-run gel with remainder of samples ⇒ See over.

RESULTS

IL1β F2/B1 Primers



OPTIMISED CONDITIONS FOR IL-1B (F2/B1)
= 2mM magnesium
56°C annealing Temp.

loading order:-

			Temp.
1)	ΦX174 Hae III Mw marker.		
2)	157 (Francis Boraiois)	mm1	56°
3)	167	mm1	"
4)	157	" 2	"
5)	167	2	"
6)	157	3	"
7)	167	3	"
8)	157	4	"
9)	167	4	"
10)	157	5	"
11)	167	5	"
12)	157	6	"
13)	167	6.	"

IL-1B FURTHER OPTIMISATION (F./B.)

⇒ Previously shown that 63°C at 1.0 - 1.5 mM magnesium produced best results. Will try.

Temperatures :- 62°/64°/66°

$MgCl_2$ ~~0.5~~ ~~1~~ ~~1.5~~ 1 / 1.5 / 2

	MASTER-MIX 1	MASTER MIX 2	MASTER MIX 3
BUFFER	2.5µl	2.5µl	2.5µl (~~15~~)
Mg	0.~~25~~ (~~2.5~~) (3)	0.~~5~~ 7~~5~~ (~~3.5~~) 4.5	0.~~75~~ 1 (6)
dNTP's	~~2~~ 4µl (10)	4µl	4µl (~~12~~) (12)
primer	2.5µl	2.5µl	2.5µl (~~15~~)
Taq	0.1	0.1	0.1 (0.6)
W-1	0.1	0.1	0.1 (0.6)
H_2O	18.8 (~~11.8~~) (~~18.8~~) (12.9)	16.05 (~~9.05~~) (9.3)	15.8 (~~7.9~~) 94.8)
TOTAL	24µl	24µl	24µl

DNA samples used Psoriasis 164 - P

174 - Q

Contents of Sto 1:

```
step 0: temp: 96.0 deg C time: 0h 5m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
begin cycle 35 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
  step 2: temp: 66.0 deg C time: 0h 1m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
  step 3: temp: 72.0 deg C time: 0h 1m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
step 5: temp:  4.0 deg C time:infinite   slope: 2.00 deg/sec  time-inc:   0 sec
total runtime (approx.):  2h37m47s
```

Contents of Sto 2:

```
step 0: temp: 96.0 deg C time: 0h 5m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
begin cycle 35 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
  step 2: temp: 64.0 deg C time: 0h 1m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
  step 3: temp: 72.0 deg C time: 0h 1m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s  slope: 2.00 deg/sec  time-inc:   0 sec
step 5: temp:  4.0 deg C time:infinite   slope: 2.00 deg/sec  time-inc:   0 sec
total runtime (approx.):  2h40m11s
```

LOADING ORDER

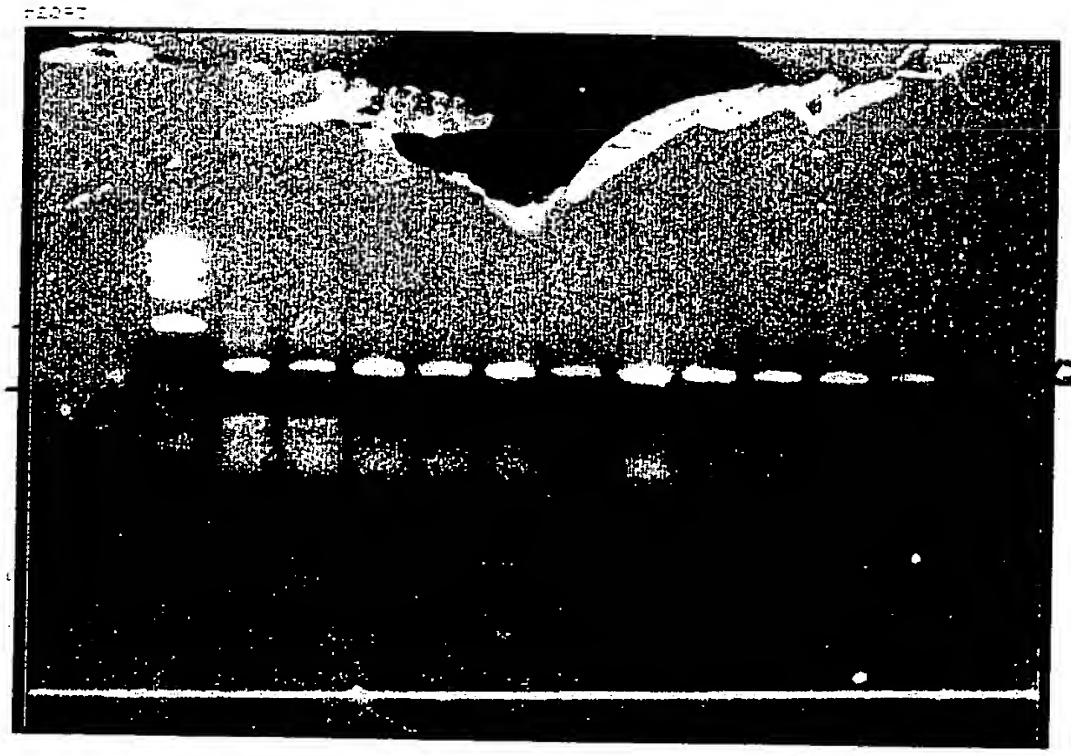
1)	ØX174 HaeIII			
2)	Psoriasis sample No.	164	mM	62°
3)	" "	174	1	62°
4)	" "	164	2	62°
5)	" "	174	2	62°
6)	" "	164	3	62°
7)	" "	174	3	62°
8)	" "	H_2O control	1	62°
9)	" "	164	1	64°
10)	" "	174	1	64°
11)	" "	164	2	64°
12)	" "	174	2	64°
13)	" "	164	3	64°
14)	" "	174	3	64°
15)	" "	H_2O control	2	64°
16)	" "	164	1	66°
17)	" "	174	1	66°
18)	" "	164	2	66°
19)	" "	174	2	66°
20)	" "	164	3	66°
21)	" "	174	3	66°
22)	" "	H_2O control	3	66°
23)	ØX174 HaeIII			

From Yesterdays disasterous Gel:-

1)	ϕX174	Hae III	
2)	157	1	56°
3)	167	1	56°
4)	157	2	56°
5)	167	2	56°
6)	157	3	56°
7)	167	3	56°
8)	157	4	56°
9)	167	4	56°
10)	157	5	56°
11)	167	5	56°
12)	157	6	56°
13)	167	6	56°

14)	157	1	60°

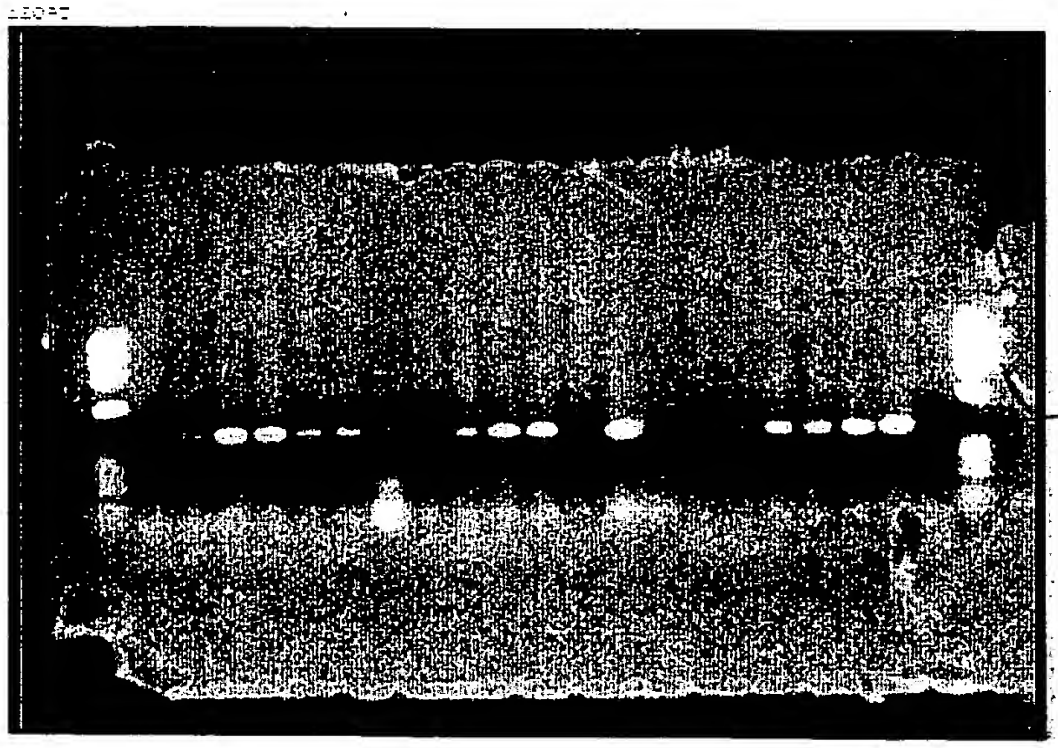
RESULTS



IL1B F2/B1 Primers

Best band = 2mM mg

56° Annealing Temp



IL1-R F1/B1

Best band = 66°C

Mg^{2+} = 2mM

wrong sized fragment must have used wrong primers

7/8/95

IL-12 - F5/B2 OPTIMISATION:

Predicted annealing Temperature 54.6°

	mix 1	mix 2	mix 3	mix 4	mix 5	mix 6
Buffer	2.5	2.5	2.5	2.5	2.5	2.5
$MgCl_2$	0.5	1	1.5	2	2.5	3.0
dNTP's	2	2	2	2	2	2
Primers	2.5	2.5	2.5	2.5	2.5	2.5
Taq	0.1	0.1	0.1	0.1	0.1	0.1
W-1	0.1	0.1	0.1	0.1	0.1	0.1
H_2O	16.8	16.05	15.3	14.8	14.8	13.8
	24µl	24µl	24µl	24µl	24µl	24µl

Try above mixes at 52°C / 54°C / 56°C

Make up enough for 8 reactions

	mix 1	mix 2	mix 3	mix 4	mix 5	mix 6
Buffer	20	20	20	20	20	20
$MgCl_2$	4	8	12	16	20	24
dNTPs	16	16	16	16	16	16
Primers	20	20	20	20	20	20
Taq	0.8	0.8	0.8	0.8	0.8	0.8
W-1	0.8	0.8	0.8	0.8	0.8	0.8
H_2O	130.4	128.4	122.4	118.4	114.4	110.4
	192	192	192	192	192	192

Contents of Sto 1:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: 52.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h58m47s

Contents of Sto 2:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
~~step 2: temp: 56.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec~~ time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h55m47s

Contents of Sto 1:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
~~step 2: temp: 56.0 deg C time: 0h 1m 0s slope: 2.00~~ deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h52m11s

loading order:

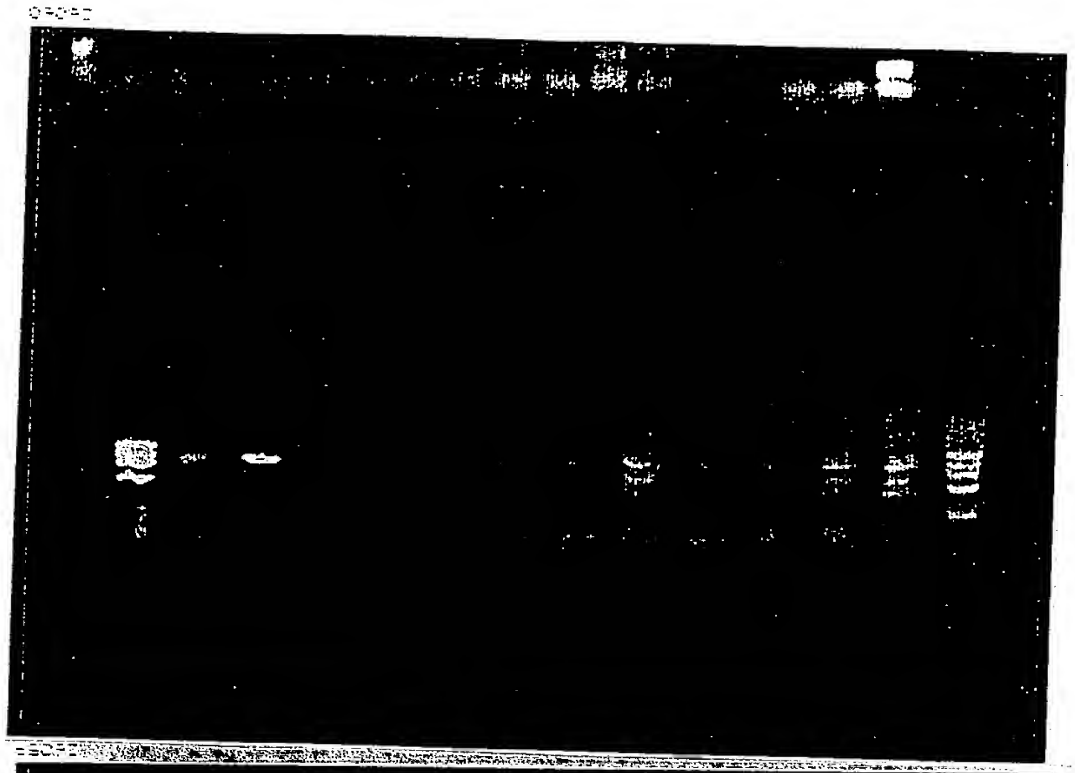
1)	8×174 marker		
2)	157	1	56°
3)	167	1	56°
4)	157	2	56°
5)	167	2	56°
6)	157	3	56°
7)	167	3	56°
8)	157	4	56°
9)	167	4	56°
10)	157	5	56°
11)	167	5	56°
12)	157	6	56°
13)	167	6	56°
14)			

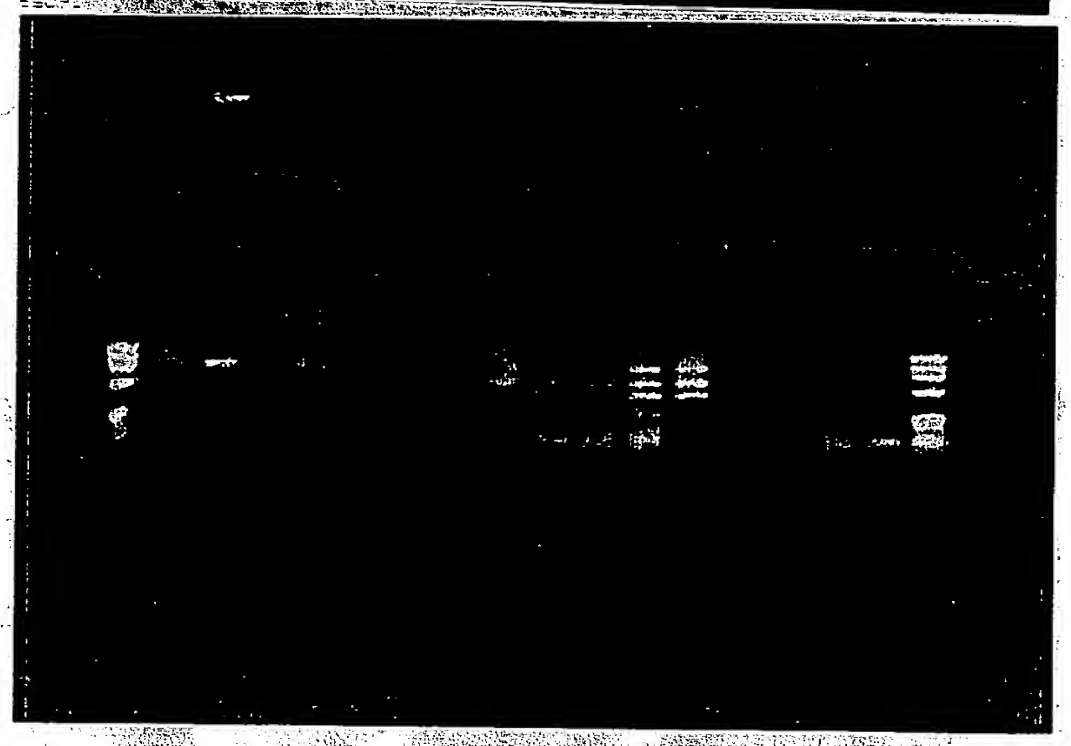
ØX174 Hae III Molecular Wt marker

1)	157	mm 1	52°
2)	167	mm 1	52°
3)	157	mm 2	52°
4)	167	mm 2	52°
5)	157	mm 3	52°
6)	167	mm 3	52°
7)	167	mm 4	52°
8)	167	mm 4	52°
9)	157	mm 5	52°
10)	167	mm 5	52°
11)	157	mm 6	52°
12)	167	mm 6	52°
	HI		
	HE		
	HS		
	HL		
	157	mm 1	54°
	167	mm 1	54°
	157	mm 2	54°
	167	mm 2	54°
	157	mm 3	54°
	167	mm 3	54°
	157	mm 4	54°
	167	mm 4	54°
	157	mm 5	54°
	167	mm 5	54°
	157	mm 6	54°
	167	mm 6	54°

Results.

54°





From these results the best bands are appearing at low $MgCl_2$ and 54°C.

⇒ Try 1mM mg
53 & 55°C
+ 2µl DNA sample.

← DISASTER GEL!,!!

IL-1B F1/B1 OPTIMISATION

Temp 62° 64° 66°

Mg 1 1.5 2.

Reagent	mm1	mm2	mm3
Buffer	2.5	2.5	2.5 (20)
Mg	0.5 (4)	0.75 (6)	1 (8)
dNTPs	2	2	2 (16)
Primers	2.5	2.5	2.5 (20)
WI	0.1	0.1	0.1 (0.8)
Taq	0.1	0.1	0.1 (0.8)
H_2O	18.8 (112.8)	16.05 (96.3)	15.8 (94.8)
	24	24	24

Use 2µl DNA Template

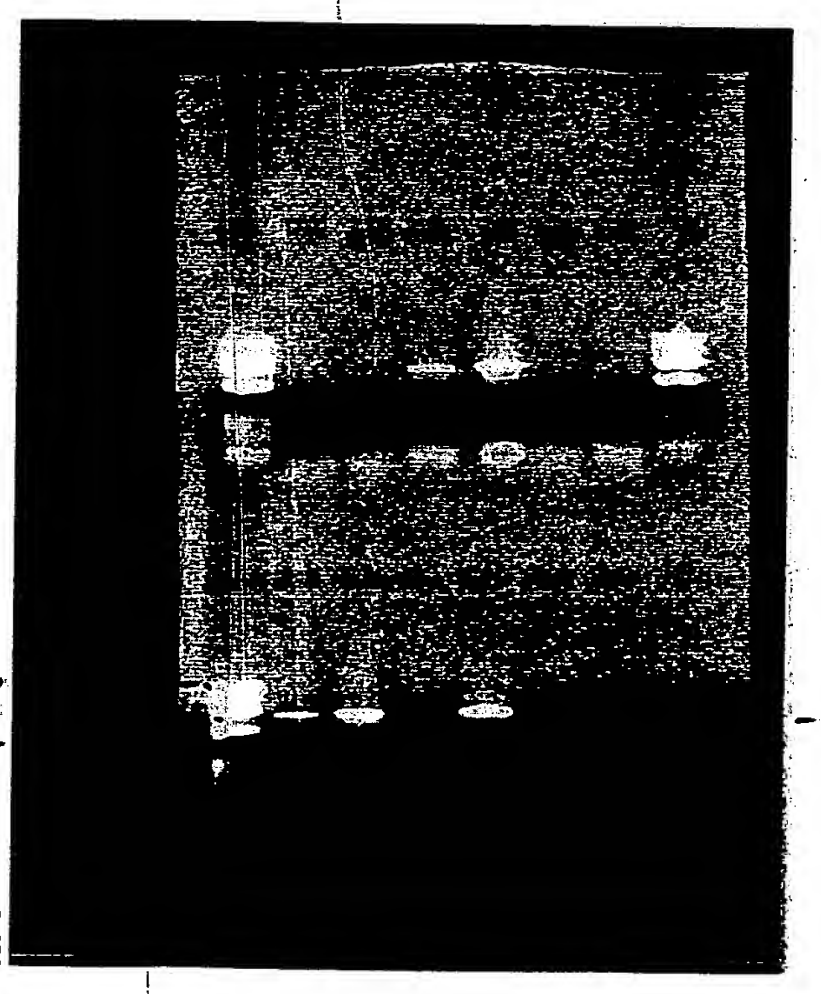
Make up enough for 8 reactions (quantities in brackets)

loading Order.

1)	~~Φ~~X174 Hae III		62
2)	157	mm 1	62°.
3)	167	mm 1	62°
4)	157	mm 2	62°
5)	167	mm 2	62°
6)	157	mm 3	62°
7)	167	mm 3	62°
~~8)~~			
8)	157	mm 2	64°
9)	167	mm 2	64°
10)	157	mm 3	66°
11)	167	mm 3	66°

H1 H2 H3

13) ~~ΦX174~~ Mw marker



- 803 bp.

B1/F1

* best sample = 66°C Annealing
2mM Mg

It seems that for the larger PCR products - using 2µl DNA. works best.

8/9/95 LL-1a P.C.R OPTIMISATION.

FS/B2 (P38 For initial expts)

Try 2mM magnesium at 58°, 55° and 60°

Reagents	Per reaction	Master mix (12)
- Buffer	2·5	30
- $MgCl_2$	1·0	24
- dNTPs	2	24
- Primers	2·5	30
- Wi	0·1	1·2
- Taq	0·1	1·2
- H_2O	14·8	177·6
TOTAL	24·0	

tubes labelled
(i) R,
(ii) S,

F4 | B2 (INITIAL OPTIMISATION PZ7)

First results not convincing - Try

1mM 2mM 3mM MgCl

Annealing Temp 58° 60° 62°.

REAGENTS	mm1	mm2	mm3	
Buffer	2·5	2·5	2·5	(20)
$MgCl_2$	0·5 (4)	1·0 (8)	1·5 (12)	
dNTPs	2	2	2	(16)
Primers	2·5	25	2·5	(20)
W-1	0·1	0·1	0·1	(0·8)
Taq	0·1	0·1	0·1	(0·8)
H_2O	16·3 (130·4)	15·8 (126.6)	15·3 (122.4	
TOTAL	24·0	24·0	24·0	

make enough for 8 reactions

L - 157
M - 167

14/11/95

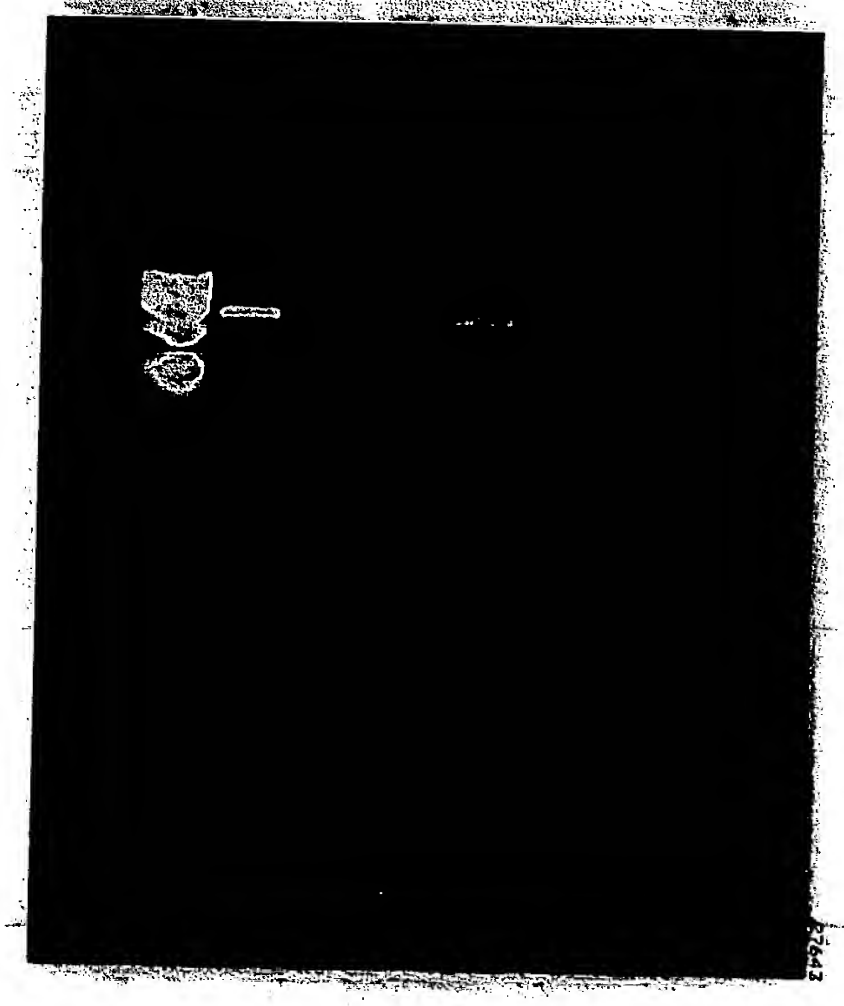
2x F1 PCR's using biotinylated primers (for mohamme

Buff	
buffer	210µl
$MgCl_2$	84µl
dNTP	168µl
Primer — F1 105	
Primer — B1 21	
W1	42
Taq	4.2
H_2O	1345µl

Do 2 samples 140 | 188 with paraffin oil
2 samples " " with Wax

Running order:-

1) QX HaeIII
2) 140 oil
3) 188 oil
4) 140 wax
5) 188 wax



20/11/95

⇒ PCR up a further (5×100µl) reactions for two samples. This time to avoid any ethanol ppt steps the sample will be run straight through a column after band purification - this will miss out any ethanol & also remove excess primer

Contents of Sto 3:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 2: temp: 66.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h37m47s

Prepare 2 samples with paraffin oil and two with wax, incase paraffin oil is interfering with the reaction

28/11/95

SSCP - Try IL1α -889 PCR cold to see if it works, - if it does redo hot & follow Jarras protocol

Contents of Sto 1:
step 0: temp: 96.0 deg C time: 0h 2m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 45 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 2: temp: 53.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 45 times
step 4: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 53.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 6: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 3h36m 8s

Date...../...../..... 53°C

Number of Samples ....10 μl

Disease.....

P'morph.....

Water	6.1 μl
Buffer	1 μl
Magnesium	1 μl
dNTPs	0.8 μl
Primers Mix	1 μl
Taq	0.05 μl
W-1	0.05 μl
Template	..... μl

IL1α -889 (30μl PCR)

each tube:

3μl 10x Buffer
2.4μl dNTPs
1.5μl 603 primer
1.5μl 604 primer
1.2μl $MgCl_2$
0.15μl W-1
0.15μl Taq
18.9μl H_2O
28.8μl

add 28.8μl to each tube + 1.2μl DNA

30/11/95

Try PCR using Angie/Nicolas Protocol - and annealing temperature at 50°C

Make up two times the volume - 5 DNA samples + 1 H_2O control.

10μl PCR 100

1μl buffer

0·8μl dNTP

0·5μl each Primer (603, 604)

0·4μl $MgCl_2$

0·05 wl

0·05 Taq

6·3μl H_2O.

8/1 1996

Biotinylated primers are correctly biotinylated - START AGAIN

Redo PCR using B primer only
When doing PCR do 45 cycles instead of 35
this will also help insure against excess primers
interfering with the reaction.

Do 4 PCR'S (5 x 100 µl reactions) 2 normal
and two cut out of gel & purified

REAGENT	VOLUME		Samples used
Buffer	~~110~~	200	
dNTP	~~88~~	160	133
primer	~~55 µl each~~	100 µM ea.	139
$MgCl_2$	~~44~~	80	173
Taq	~~4 µl~~	8	179
W-1	~~4 µl~~	8	
H_2O	~~695.2 µl~~	1264	
	1100 µl		

Contents of Stp 1:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 45 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: 60.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 45 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time: infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 3h28m21s

gels PCR

Primer	50 each
$MgCl_2$	40
Taq	5
W-1	5
H_2O	630 µl

- PCR DID NOT WORK - TRY AGAIN WITH FRESH REAGENTS esp dNTP

21/1/96

Re-dissolve returned primers in 150µl H_2O - measure O.D.

5µl → 995µl H_2O. → O.D.

SAMPLE	A320	A280	A260	280/260	260/280	PROTEIN	NUCLEIC ACID
1.0000	0.0036	0.1146	0.1865	0.6065	1.6488	33.653	7.5100
2.0000	0.0048	0.1625	0.2776	0.5782	1.7296	38.189	11.478

B1 - dilution factor =

$$A_{260} \times DILN \times 0.033 / WM \times 10^6$$

$$= \frac{(0.1865 \times 200 \times 0.033 / \frac{7255}{6787})\,10^6}{20}$$

1:9 dilution

B2 $\frac{(0.2776 \times 200 \times 0.033 / \frac{8269}{7775})\,10^6}{20}$

= 1:9 dilution

1:11 dilution

Do ILIB/2 PCR's

	M1	M2
Buffer	200	200
$MgCl_2$	80	80
dNTPs	160	160
Primer	100µl F2 / B1	100 F5 / B2
Taq	8	8
W-1	8	8
H_2O	1264	1264
	2,000	2,000

4 reactions @ 100µl x 5

Use samples 115 } 1,1 for +3953
151 }

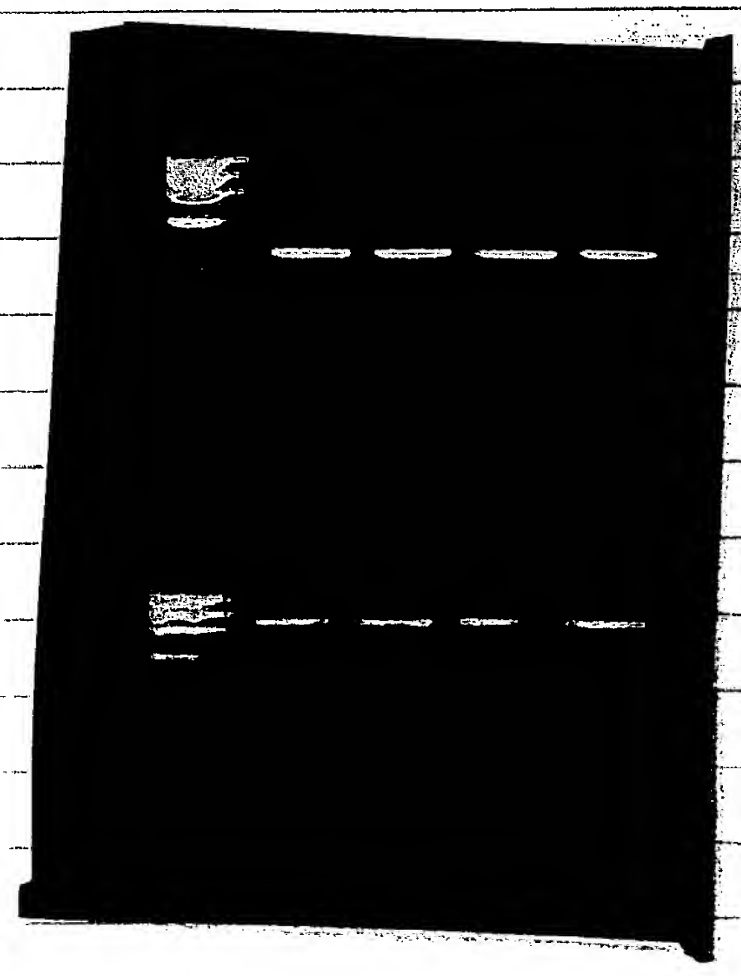
121 } 2,2 for +3953
126 }

23/1/96

Running order:-

1) QX Hae III
2) 115 F2
3) 151 F2
4) 121 F2
5) 126 F2
6) 115 F5
7) 151 F5
8) 121 F5
9) 126 F5
10) ~~QX~~ H_2O control
11) QX Hae III

Run 10µl on 1% Agarose gel



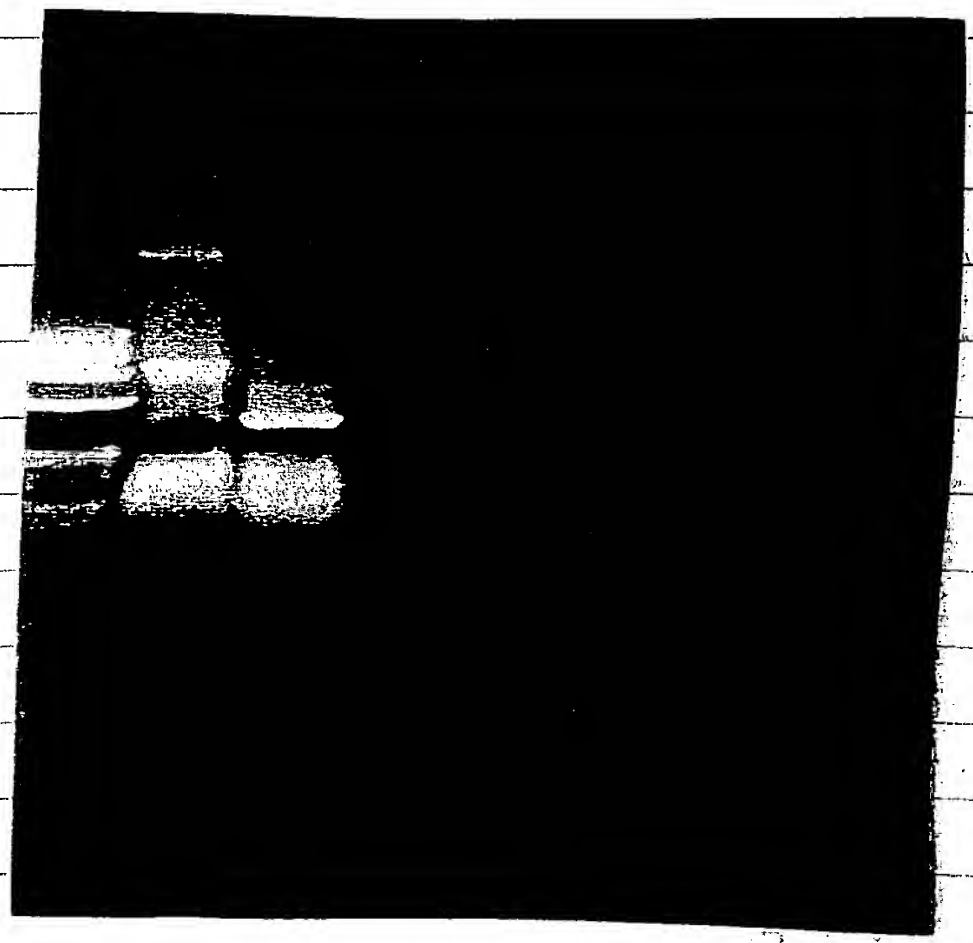
Do 115 and 121 for each Normally
- 151 and 126 extract band from gel to remove primers

RUNNING ORDER - (ON THE GEL)

F5 151
F2 151
F2 126
F5 126

- Bands were cut out of the gel, and purified through glass wool and ethanol precipitat

Samples were made single-stranded using dynabeads - samples of of Supernatants were kept & run on a 1% Agarose gel.



RUNNING ORDER

1) QX Hae III
2) 1st Wash F2 121
3) " " F5 151
4) ~~1st wash~~ SS DNA F2 121
5) SS DNA 121
6) 1st Wash F2 121
7) " " 115
8) SS DNA F5 151

We can see from the previous gel picture that for samples which had excess primer removed by running samples down the gel showed no loss of sample on 1st wash and a clear band appeared on the lane encorporating ss DNA - The band may not be very bright but it is well known that ss DNA does not encorporate ss DNA so well

⇒ Try Sequencing F5 151 DNA.

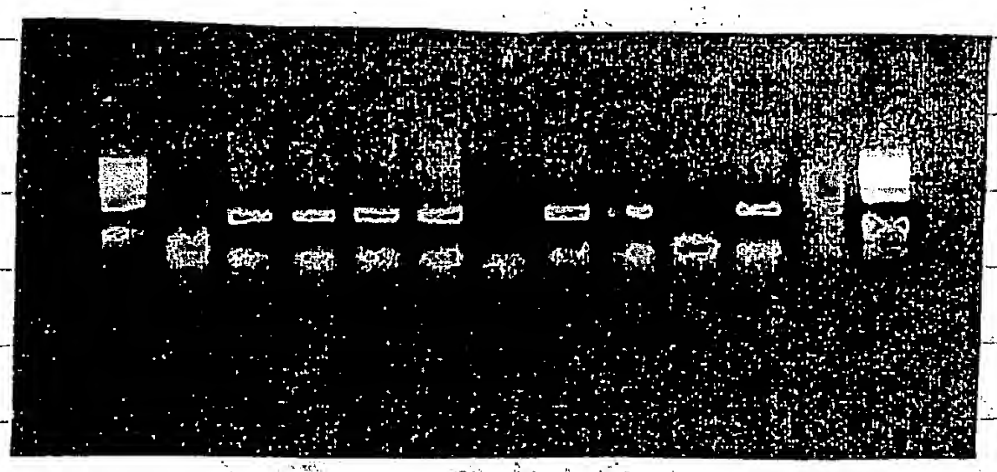
23/1/96

Sequencing of PCR product (not biotin) is now possible and very successful.

To prepare PCR's for samples F1 - B1 & F5 - B2 5 Tag 1,1 and 5 × Tag 2,2

⇒ enough for 160 reactions each

⇒ Reagent	MM1	MM2.
Buffer	500µl	500µl
$MgCl_2$	200µl	200µl
dNTP	400µl	400µl
Primer	250µl each	250µl each
W-1	20µl	20µl
Taq	20µl	20µl
H_2O	2960	2960
	4600	4600

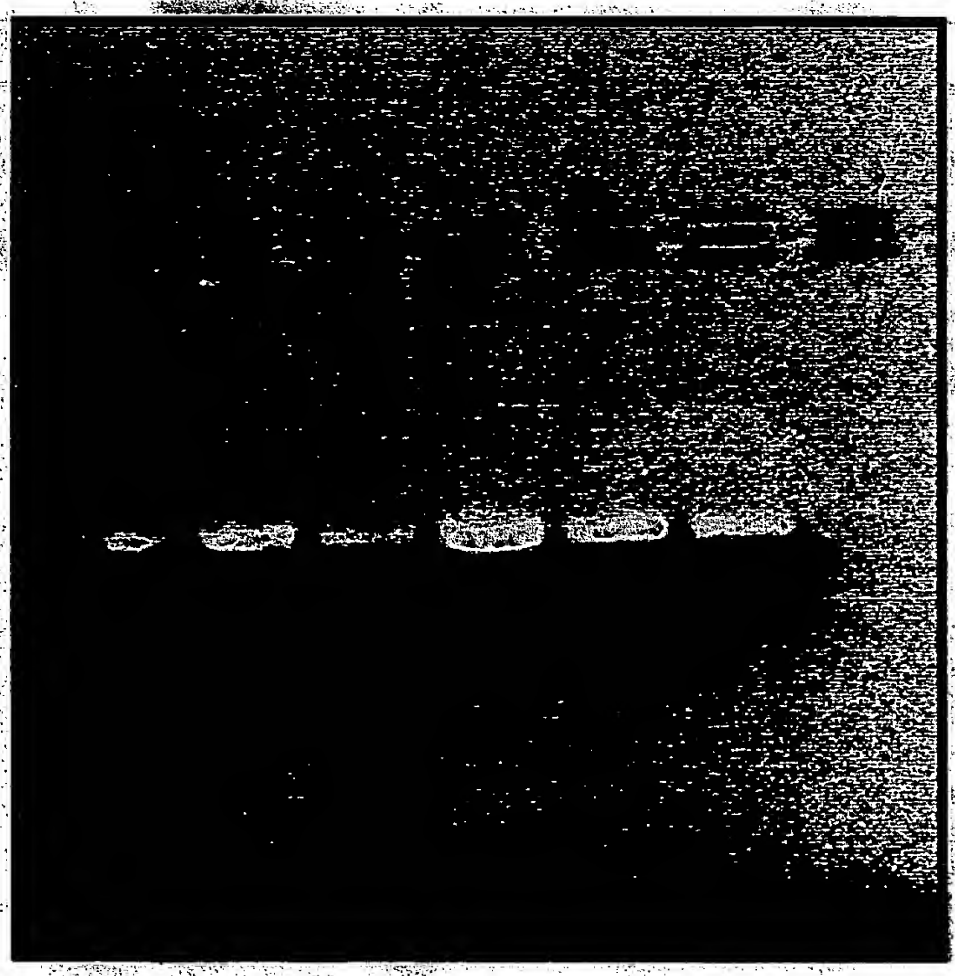


Running order

1) 115
2) 121 ⌐
3) 126 ⌐
4) 144 ⌐
5) 165 ⌐
6) 113
7) 139 ⌐
8) 157 ⌐
9) 112
10) 157 ⌐
11) H_2O

Contents of Sto 1:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0
begin cycle 45 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc:
  step 2: temp: 56.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc:
  step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc:
end cycle 45 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 s
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 s
total runtime (approx.): 3h36m 1s

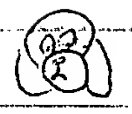
Take all samples that worked, pool their DNA and ethanol extract - then run on a gel and band purify the sample for sequencing



← Run on a gel to check samples had not been lost by above process

1/2/96

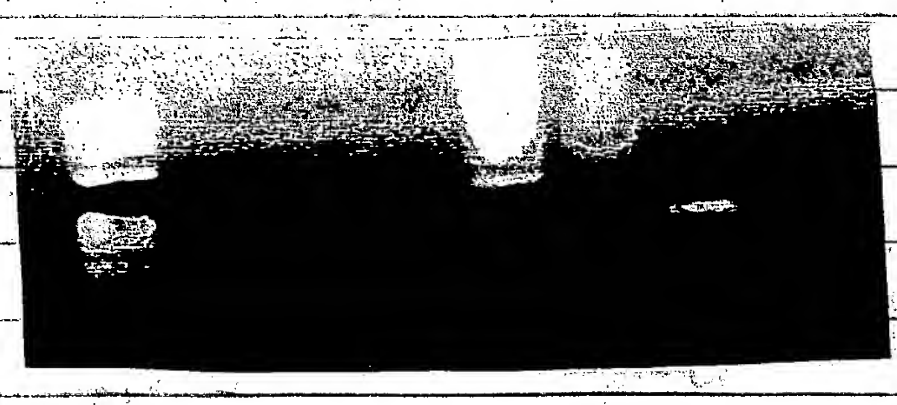
MUTS

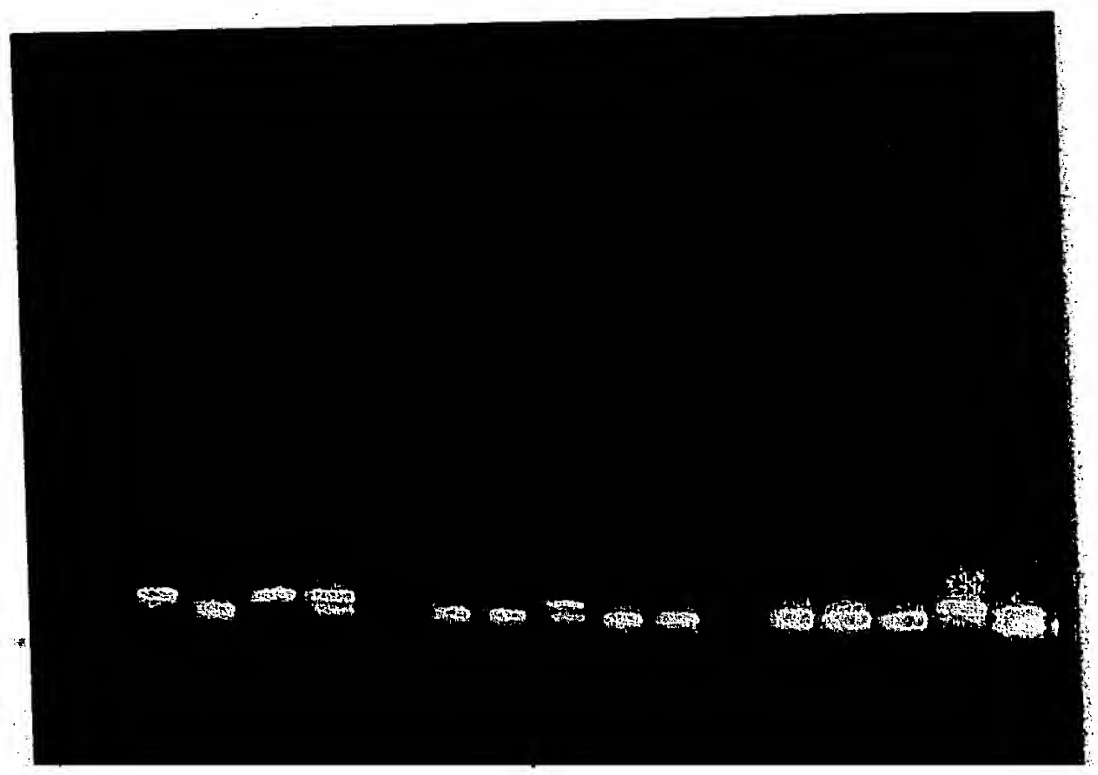


0/

96 °C - 5'
37 °C - 5'
X3

1) Take 50µl DNA (genomic) [4µl X 50]
2) Add 140µl buffer (TRIS HCL PH 7.5) PCR BUFFER - to make volume ~~200~~ 190 µl
3) Add 10µl beads
4) Agitate gently by flicking - incubate @ 37°C - ½ hr with shaking
5) Centrifuge briefly
6) Apply makmet
7) Wash in 200µl Wash buffer X2
8) Add buffer from step 2 - heat - 75°C-15'
   Remove supernatant - & PCR - Using F1/B1 primers as before! - Run on gel to see if anything's there!





1/2/96

- Do a 100µl PCR reaction with Alisons Taq primers expected fragment size

- also Do 100µl PCR with IL1β F2-B, expected fragment size - 400 bp

Taq

Reagent	20µl Volume	MM	
Buffer	~~1.2~~ 2	20	24
$MgCl_2$	2	20	24
dNTP	1.6	16	19.2
W1	0.1	1	1.2
Taq	0.1	1	1.2
Primer	2-mix	20	24
H_2O	12.2	122	146.4

1.6 / 12 / 3 / 120 / 160 / 20 / 192

IL1β

Reagent	Volume	
Buffer	15µl	20µl
$MgCl_2$	6µl	8µl
dNTP		16µl
Primer		10µl each
Taq		0.8µl
W-1		0.8µl
H_2O		120µl

10µl template

step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 45 times:
  step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 2: temp: 56.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
  step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 45 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 3h36m 1s

5/2/96

Samples for sequencing :-

1 - 121
2 - 126
3 - 157
4 - 151
5 - 144
6 - 139
7 - 165

- Give Hazel F2 primer to sequence half of each of the samples. - Taq FS sequencing

Do PCR's as before ←

Contents of Sto 2:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 45 times:
step 1: temp: 94.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: 56.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 45 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 3h36m 1s

Contents of Sto 1:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 45 times:
step 1: temp: 96.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: 64.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 45 times
step 4: temp: 72.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 3h24m27s

Do PCR's on 1L1d/β for direct sequencing from PCR product.

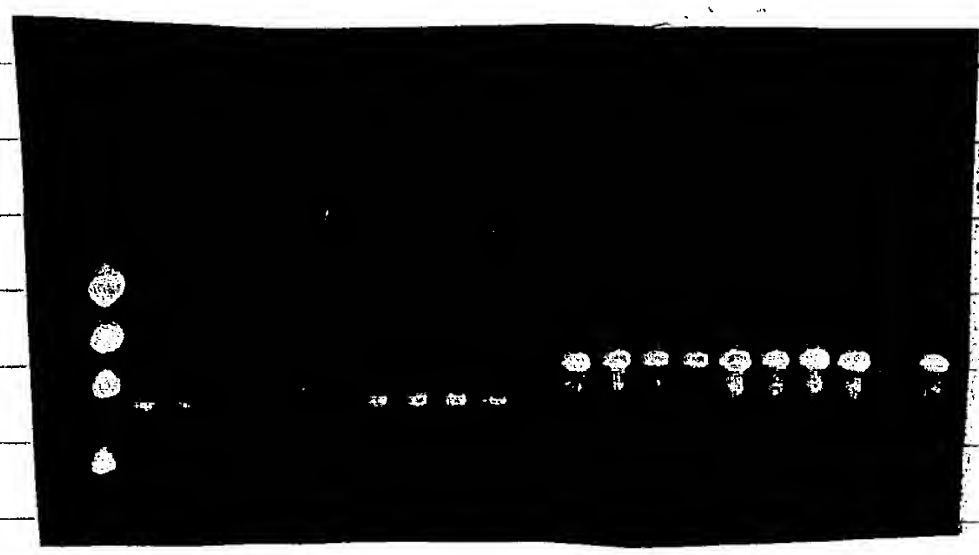
Do PCR with F5/B2 and sequencing using both F6/F4 primers

F1/B1 Pcr. - again both F1/F2 primers

	REAGENT	Volume	Volume
10 reactions x 10 = 100	buffer	500µl	500
	dNTP's	~~200~~ 400	400
	$MgCl_2$	200	200
	primer	250 each	250 each
	Taq	20µl	20µl
	W-1	20µl	20µl
	H_2O	2960µl	2960µl
		4600µl	4600µl

Running order:-

1) QX HaeIII
2) 115 F⁻
3) 151
4) 157
5) 165
6) 174
7) 121
8) 126
9) 189
10) 144
11) 152
12)



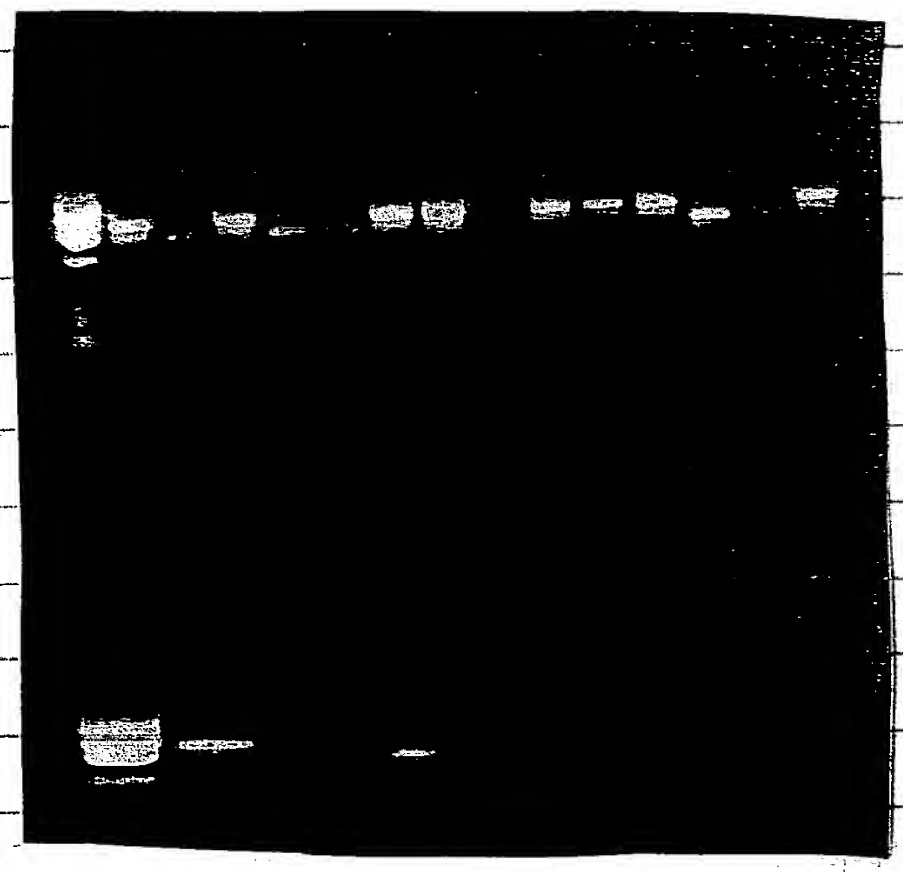
3/2/96

- Run PCR products on gel - band extract and ethanol precipitate - Give to hazel to sequence

Gel to check products are still there

Running Order

1) ΦX Hae III marker
2) F5 174
3) F1 126
4) F5 151
5) F1 151
6) F1 174
7) F5 152
8) F5 157
9) F1 139
10) F5 121
11) F5 139
12) F5 165
13) F1 152
14) F1 165
15) F5 115
16) F5 126
17) F1 157
18) F1 144
19) F1 115

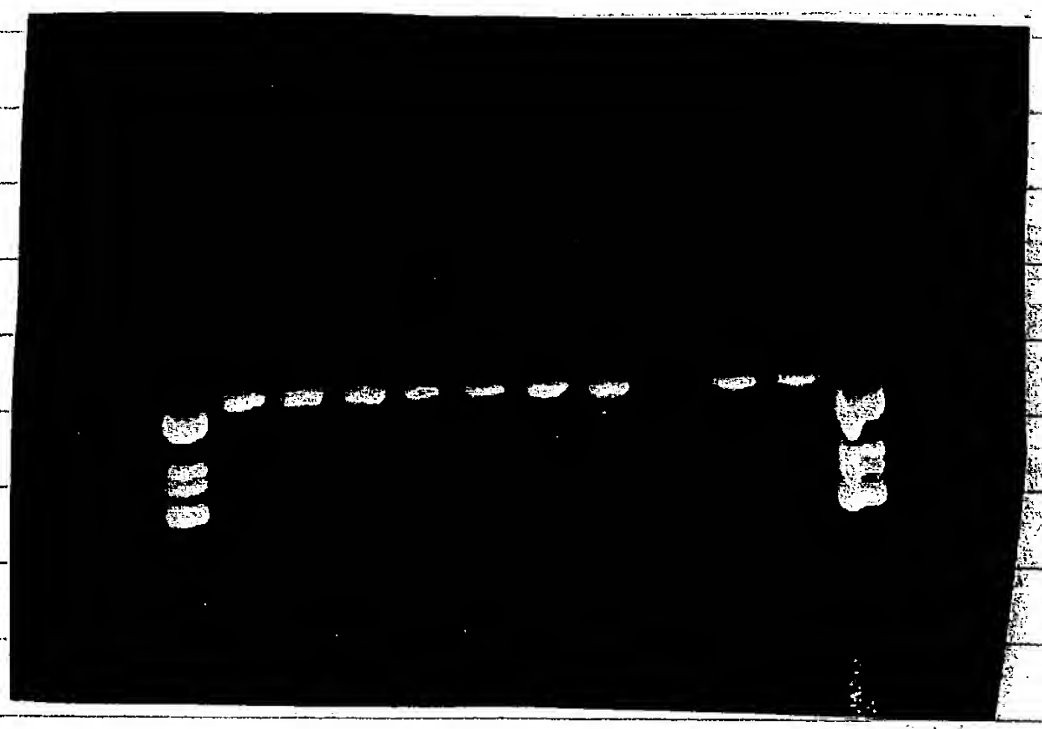


10/2/96

Do Biotinylated PCR's for 5x 1,1
5 x 2,2.

REAGENT	VOLUME
buffer	500µl
$MgCl_2$	200µl
dNTP	400µl
Primer	250µl each (used 200 F1 100 BBi)
N-1	20µl
Taq	20µl
H_2O	2960µl

Run samples on a 1% agarose gel



RUNNING ORDER

1) QX Hinf
2) 115 F1 / Bb1
3) 151 F1 / Bb1
4) 157 F1 / Bb1
5) 165 F1 / Bb1
6) 174 F1 / Bb1
7) 121 F1 / Bb1
8) 126 F1 / Bb1
9) 189 F1 / Bb1
10) 144 F1 / Bb1
11) 152 F1 / Bb1

11/12/06

Pool all 5 x100 µl's and ethanol precipitatate resuspend in 20µl for running on TAE gel & band extract

Running Order.

1) 139
2) 151
3) 126
4) 144
5) 115
6) 121
7) 157
8) 174
9) 152
10) 165

After band extraction, Samples were ethanol precipitated and resuspended in 40µl H_2O. - 1µl of sample was run on a 1% agarose gel to check that I have not lost them during ethanol precipitation.

14/2/96

Do IL-1 Taq PCR on 50 patients (4×100µl.) for contol experiments using nuts 5 beads
⇒ Worked OK.

Mohammeds Solutions -FIL57
-FI-174
FI-152
FI-121
-FI-139

1% = 1ml/100
0.005% =

0.005%

5µl ⇒ 100mls
0.5 → 10mls
20µl → 10mls
1µl → 40

15/2/96

MUTS - New Protocol

- Carry out reaction in 10mM Tris-HCl (Not 1M)
- Put ~~1/2~~ 0.05% tween 20 in buffer rather than BSA.
- ensure buffer is FILTERED through 0.45μM
- Binding occurs at room temp - Not 37°C
- Wash beads with 10mM T/HCl (No tween)

CONTROLS

(1) Beads - No DNA - boil, remove 5ᴺ - PCR as a H_2O control.

(2) One @ 37°C and one at Room temp

7 beads with no bsa - but tween more OK

REACTION BUFFER

- 1M Tris HCl pH 7.5
- 250mM $MgCl_2$
- 5mM dTT
- 0.5mM EDTA

} 50× ⇐ PCR buffer! + add $MgCl_2$ Equal Volume

Block beads with 5mM P.V.P.

2g → 10mls

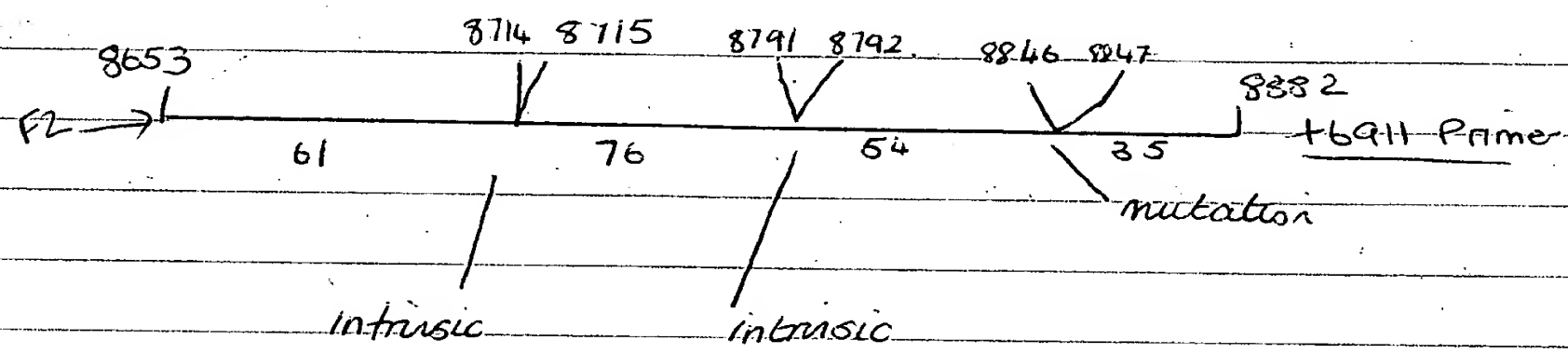
- Again samples were heated to 75°C for fifteen minutes and supernatant removed immediately

21/2/96

Sequencing has revealed a possible G-C Change

Design method for screening possible polymorph

- No enzyme (except TspRI) cuts at the altered site. Therefore a cut site must be engineered - one possibility is to engineer a cut site for HinfI in a primer. There are 2 intrinsic HinfI sites + the engineered one.

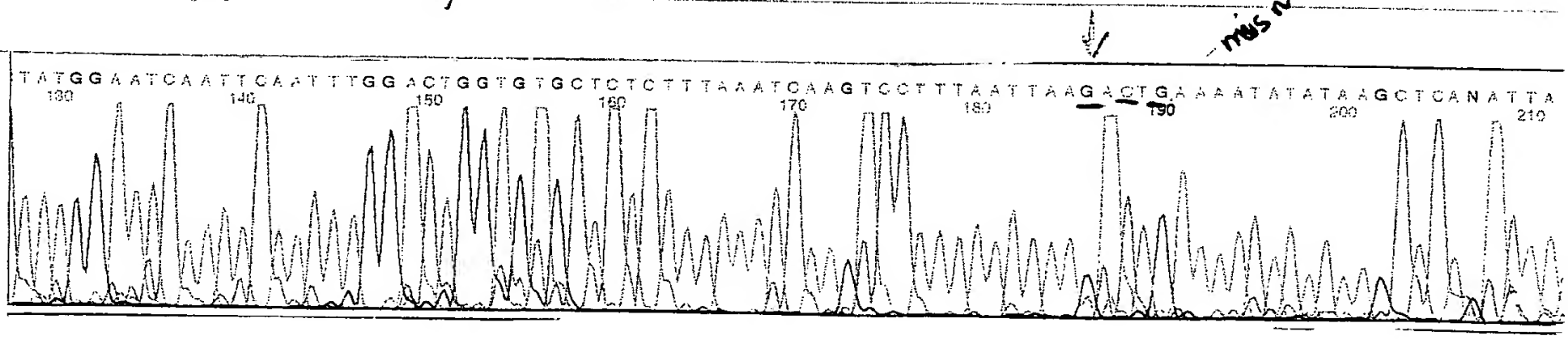


EXPECTED BANDING PATTERNS

	GG	CC	GC
89	—		— faint
76	—	—	—
61	—	—	—
54		—	— faint
35		—	— faint

Design oligo which creates the hinf site.

Because,



21/ 2/96

Colum £3 MM828
Seq: Seq03
Overall: 50.5
ASWY: 98.0

Num	Base	ASWY
2	C	100.0
3	C	98.5
4	A	96.0
5	T	97.0
6	T	97.6
7	T	98.0
8	A	98.0
9	A	98.0
10	A	97.9
11	T	98.0
12	A	98.1
13	A	97.4
14	T	97.6
15	C	97.8
16	T	97.9
17	G	98.0
18	A	98.1
19	G	98.2
20	C	98.3
21	T	98.2
22	T	97.4
23	A	97.5
24	T	97.7
25	A	97.8
26	T	97.8
27	T	97.9
28	T	98.0
29	T	98.1
30	T	98.1
31	T	97.7
32	G	97.8
33	A	97.9
34	G	98.0
35	T	98.0

SEQUENCE: t6911 Primer

5' TG A GTT TTA TAT ATT ATT

CGA GTC TAA TAA ATT TAC C 3'

Predicted annealing temperature
57°C

(Using hyperoad)

Column 1 14:53:21 ,23/ 2/96

Run ID : MM831
Cycle : 002 UMOL
End Proc: End CESS (DMT = Off)
Sequence: Seq01

Carol

Average
Step-wise
Yield : 98.8
Total bases = 35
A= 11, G= 4, C= 5, T= 15, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 10706.0

5'> CCC ATT TAA ATA ATC TGA GCT TAT ATA TTT TGA GT <3'

Ethanol precipitate primers and set up optimisation of PCR's.

OPTIMISATION STRATEGY

	2mM	3mM	4mM	5mM
Buffer	35	35	35	35
$MgCl_2$	14µl	21µl	28µl	35µl
dNTP	28µl	28µl	28µl	28µl
Primer	35µl	35µl	35µl	35µl
W-1	1·4	1·4	1·4	1·4
Taq	1·4	1·4	1·4	1·4
H_2O	207·2	207·2	207·2	207·2

2 samples - 3 temperatures + 1 H_2O
= 7 tubes

$A_{200} = 0.2411$

$$\left(A_{260} \times \text{dil}^n \times 0.033/Mw \times 10^6 \right) = \text{Air factor}$$

$$\left(\frac{0.2411 \times 200 \times 0.033}{10706} \right) \times 10^6 = \text{Dilution factor}$$

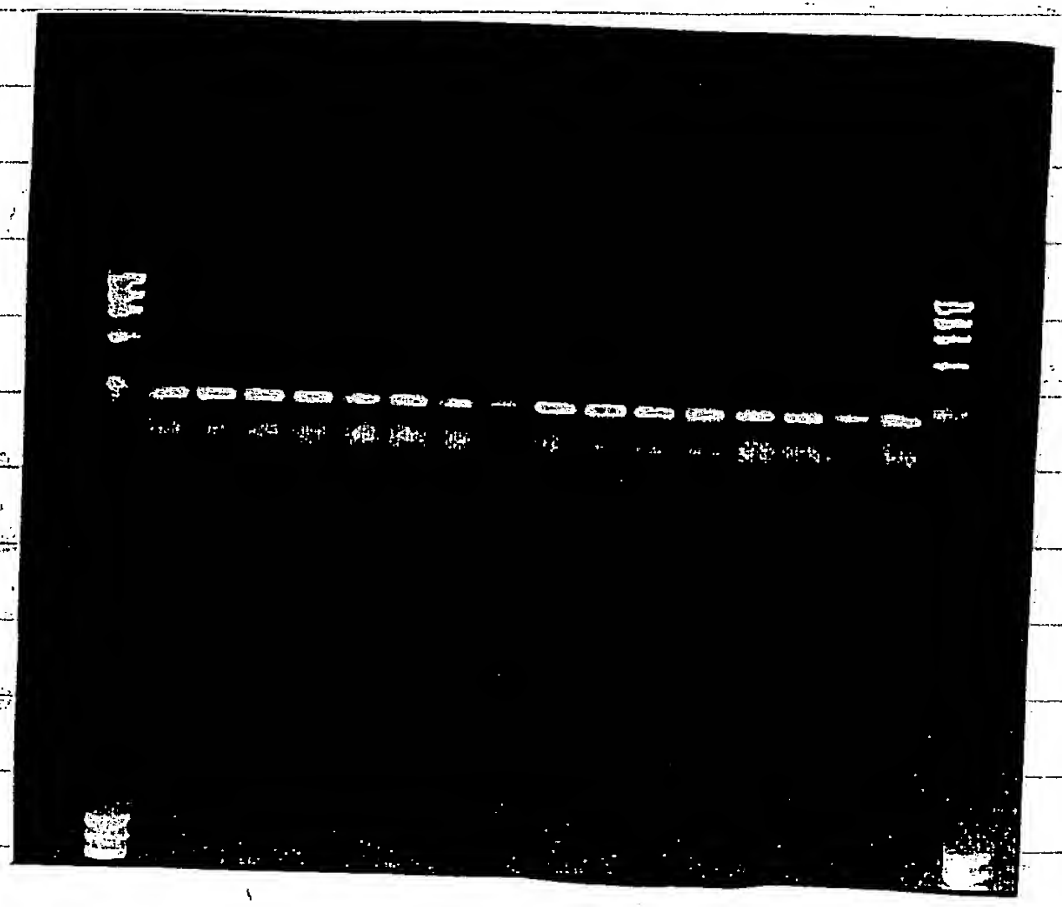
$$159 \text{ mg/ml}$$

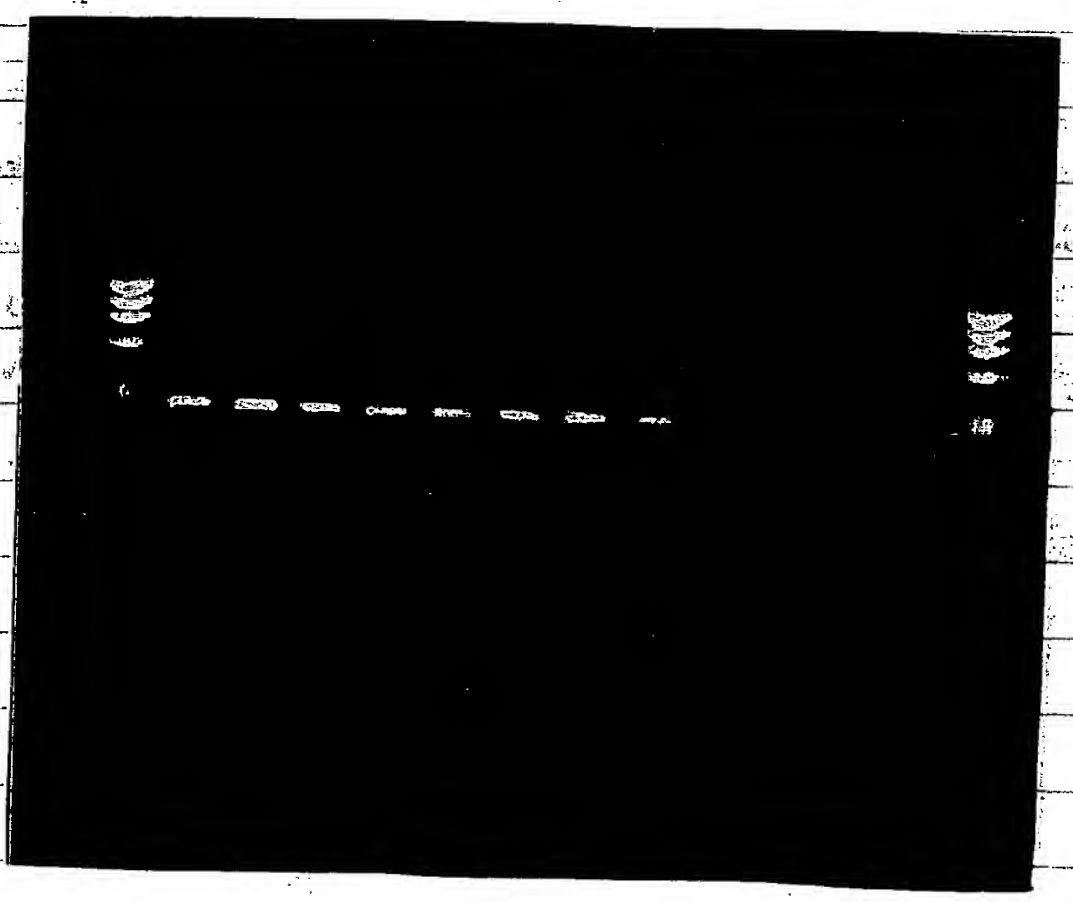
$$0.148 \text{ mM}$$

$$1 : 7.43$$

RUNNING ORDER:

1)	QX Hae III		Run 10µl on a 1% Agarose gel
2)	255	1 → 4 mM $MgCl_2$	54°C
3)	256		
4)			
5)			
6)	255	1 → 4 mM Mg	56°C
7)	256		
8)			
9)			
10)			
11)	255	1 → 4 mM $MgCl_2$	
12)		↓	
13)	256	—	58°C
14)			
15)			
16)	H_2O - 1		
17)	- 2		
18)	- 3		
19.	- 4		





- See p129 for Running order

all Magnesium concentrations worked at all 3 temperatures ∴ Do 30 x 50µl PCR's on

10 x 1,1
10 x 1,2
10 x 2,2

} for Taq Polymorphism

ie: 31 tubes

Reagent	Volume
Buffer	155µl
$MgCl_2$	62µl
dNTP	124µl
Primer	87.6 each
W-1	6.2µl
Taq	6.2µl
H_2O	917.6
	1426.

4µl template
+46 µl mastermix

Carry out reaction @ 56°C

Digestions - 0.5 (2.5)µl enzyme per tube
~~3660~~ tubes

7.5µl enzyme
60µl buffer
} 2.25µl ↓ 20µl

15µl enzyme
120µl buffer
}

leave to digest overnight @ 37°C

Run samples on a 9% polyacrylamide gel @ 200V for 30 minutes

Samples used:-

Gel 1

	TAQ	+3845	
112	1,1	-	CC
113	1,1	-	-
114	2,2	-	GG
115	1,1	-	CC
116	1,2	-	GC
117	1,1	-	CC
118	1,2	-	CG
119	1,1	-	-
120	1,1	-	CC
121	2,2	-	GG
122	1,2	-	GC
123	1,2	-	GC
124	1,2	-	GC
125	1,2	-	GC
126	2,2	-	GG

Gel 2

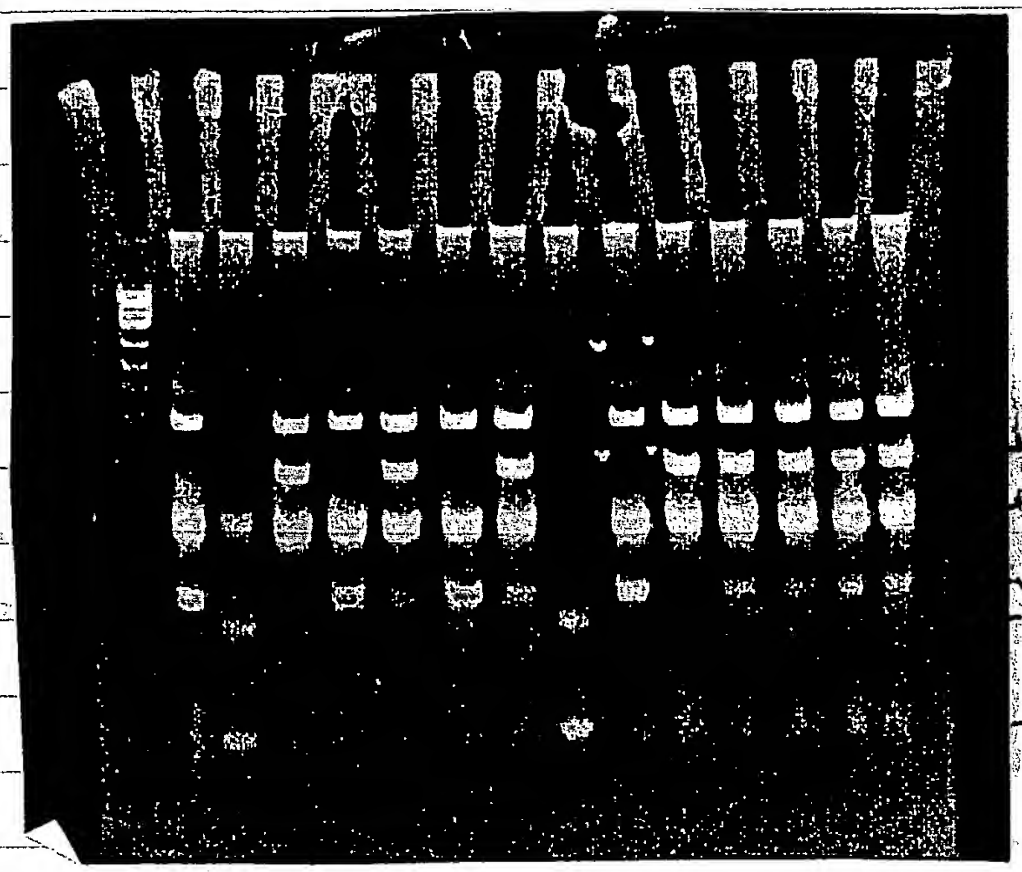
	TAQ	
127	1,1	- CC
128	1,2	- GC
129	1,1	- CC
130	1,2	- GC
132		CC
133		GC
134	1,2	GG
135	1,2	GC
136	1,1	CC
137	1,2	GC
138	1,1	CC
139	2,2	GG
140	1,1	
141	1,1	
H2O		

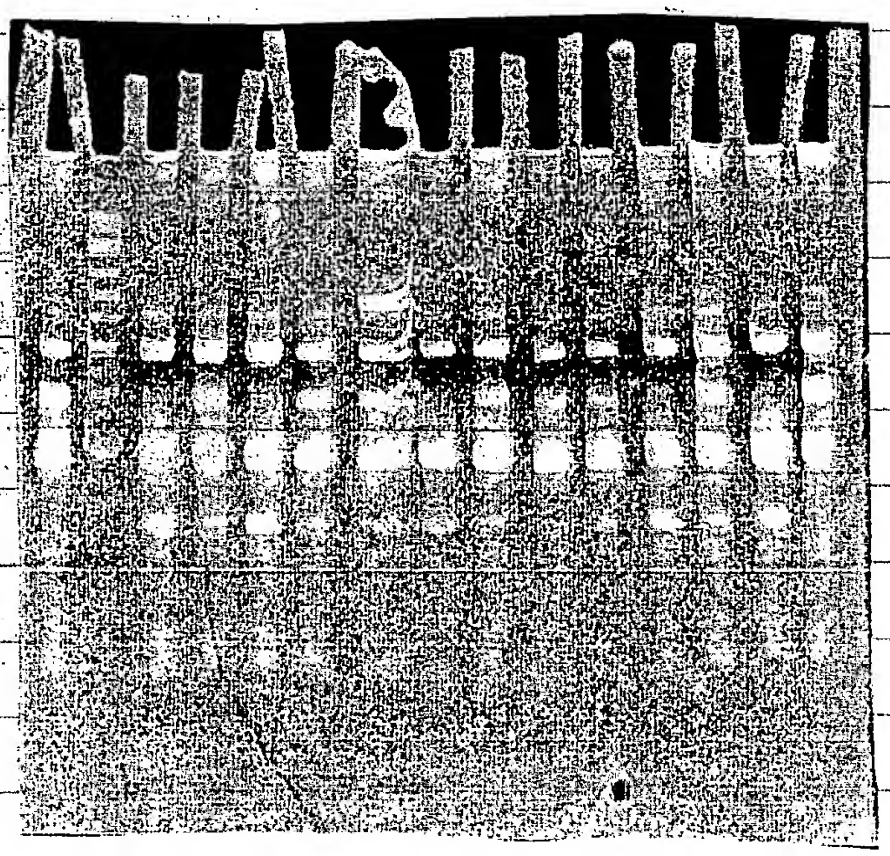
12 x 1,1

11 x 1,2

4 x 2,2

Thus it appears that Allele (G) of my polymorphism is 100% associated with allele 2 of alleles Taq - and C is 100% linked to allele 1





FUTURE PLANS FOR +8845 POLYMORPHISM

- Fusions of AU Rich region to β globin

Normal mRNA ½ life
= 17hrs

- Band shift assays with both mRNA's incubated with nuclear extract.

1st - ASTA using Adeels Taq-man System

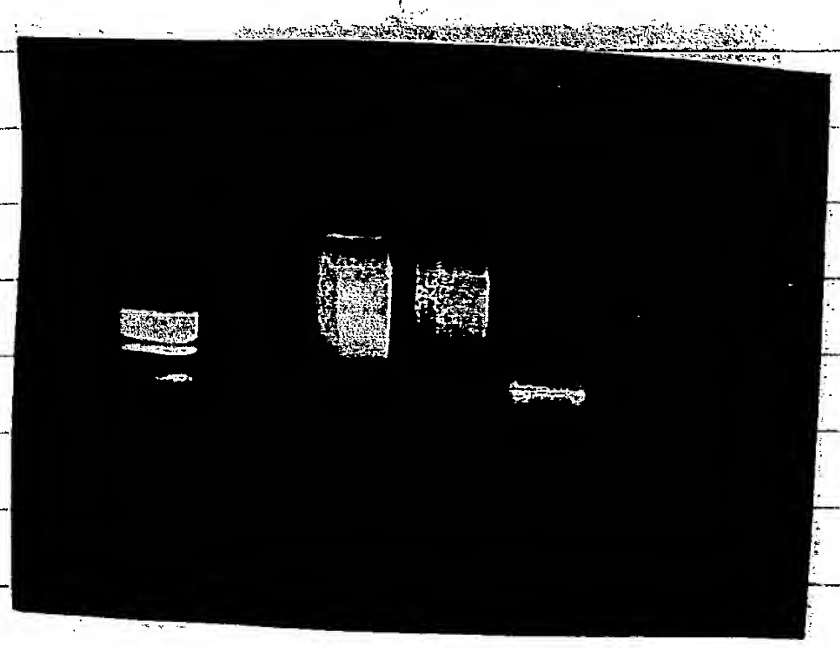
1/3/96

Set up Taq PCR again

~~12~~ 2 x 100 µl PCR's > 4 MUTS Reactions

REAGENT	VOLUME
Buffer	40
$MgCl_2$	40
dNTP	32
W-1	2
Taq	2
Primer	40
H_2O	244

- add 8µl template for each :-



- Obviously this has not worked - Why am I getting such smears

CONTROL EXPTS

1) Band extract DNA before putting ove the column
2) Try Re-PCR'ing the PCR product to see if I get the smears then
3) Run on acrylamide & stain for protein

8645
1941
6904
20 of each

Plan Expts - PCR-up 1,1 / 2,2 / 1,2 } check that they are same for mice

- How much blood?
- Time scale?

1 LIB peaks @ 6 hr
+ 18-24 hr

37° 15'

- Anti CD3 / CD19 beads - use these to pull off pure cell types
- Standard curve

Taqman 5 / 10 / 15 / 20 cycles

Quantitation of steady state mRNA levels
⇒ Stability or accumulation of mRNA

eosinophils - acidic - stain eosin 145
basophils basic
neutrophils

mononuc
- lymphocytes - from lymph - nucleus almost complete cell diameter dense chromatin
- monocytes - bean kidney shaped nucleus much less dense than lymphocytes - because DNA is ~~not~~ active

100 cells - 30 neutro
- 50 lymo
- mono

5/3/96

Do Taq PCR for further MUT-5 expts - as previously set out (P115)

RUNNING ORDER

1) QX1174 Hae III
2) Taq
3) Taq
4) H_2O contd

Also F5 - BB_2 PCR for sequencing of IL1 2'

Running Order ~~QX Hae III~~

2) 165
3) 174
4) 149
5) 115
6) 157
7) 152
8) 139
9) 126
10) 121
11) 144
12. H_2O

DO 40 x 25µl reactions to confirm linkage of + ?? to Taq polymorphism

Taq PCR

3 samples from muts
1 positive control ⇒ 20µl reaction
1 H_2O control

REAGENT	VOLUME
Buffer	8
$MgCl_2$	8
dNTP	12.8
Primer	8 µl
Taq	0.8
W-1	0.8
H_2O	97.8

RUNNING

1) Q x Hae III
2) PVP 50
3) PVP 100
4) TWEEN
5) +'ve control
6) H_2O control

Do ~~96~~ 48 x 25µl PCRs of new Polymorphism PCR

REAGENT	VOLUME
Buffer	120
$MgCl_2$	~~96~~ 48
dNTP	96
Primer	4 120
Taq	4·8
W-1	4·8
H_2O	710·4
	110·4

2µl template per reaction

* Using the plate, some of the samples have evaporated.

- For digestion do 1~~2~~ with ~~10~~ 10 µl of sample digested with 0·5 µl enzyme overnight
and a further 1~~2~~ with 0·25 µl enzyme overnight

MM1	MM2
6µl enzyme	3µl enzyme
18µl buffer	18µl buffer

7-19 0·5µl enzyme / 10µl
21-36 0·25µl enzyme / 10µl

6/3/96

Re-do ~~FS~~-B62 PCR's on samples :-

152

139

126

121

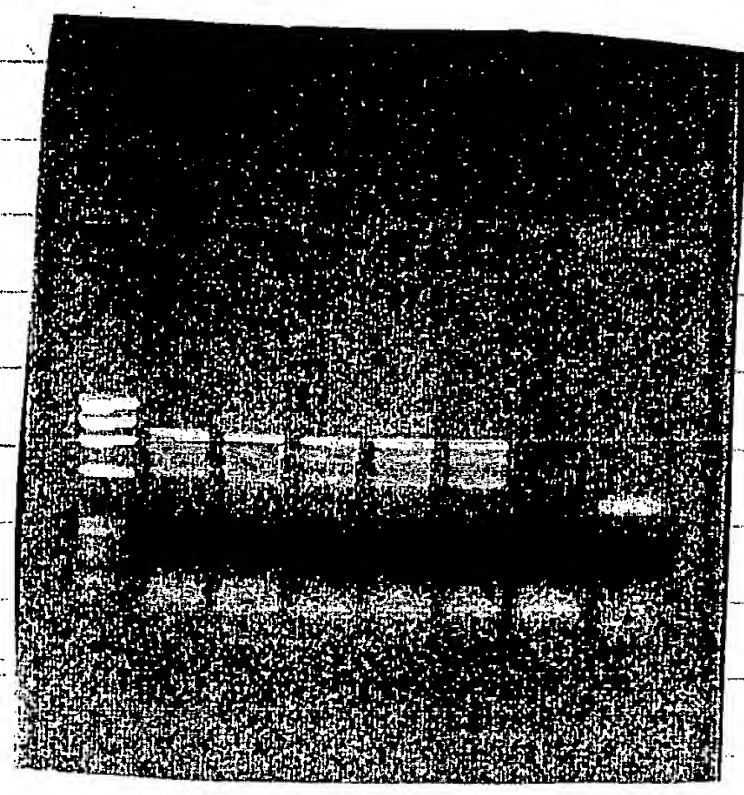
144

REAGENT	VOLUME
Buffer	130
$MgCl_2$	52
dNTP	104
Primer	65µl each / 65µl each
W-1	53 µl
Taq	5·2µl
H_2O	769·6
TOTAL	1196

Add 8µl template to each PCR.

RUNNING ORDER :-

1) ΦX HaeIII
2) 126
3) 139
4) 144
5) 152
6) 121
7) H_2O
8) 115 SS.



130

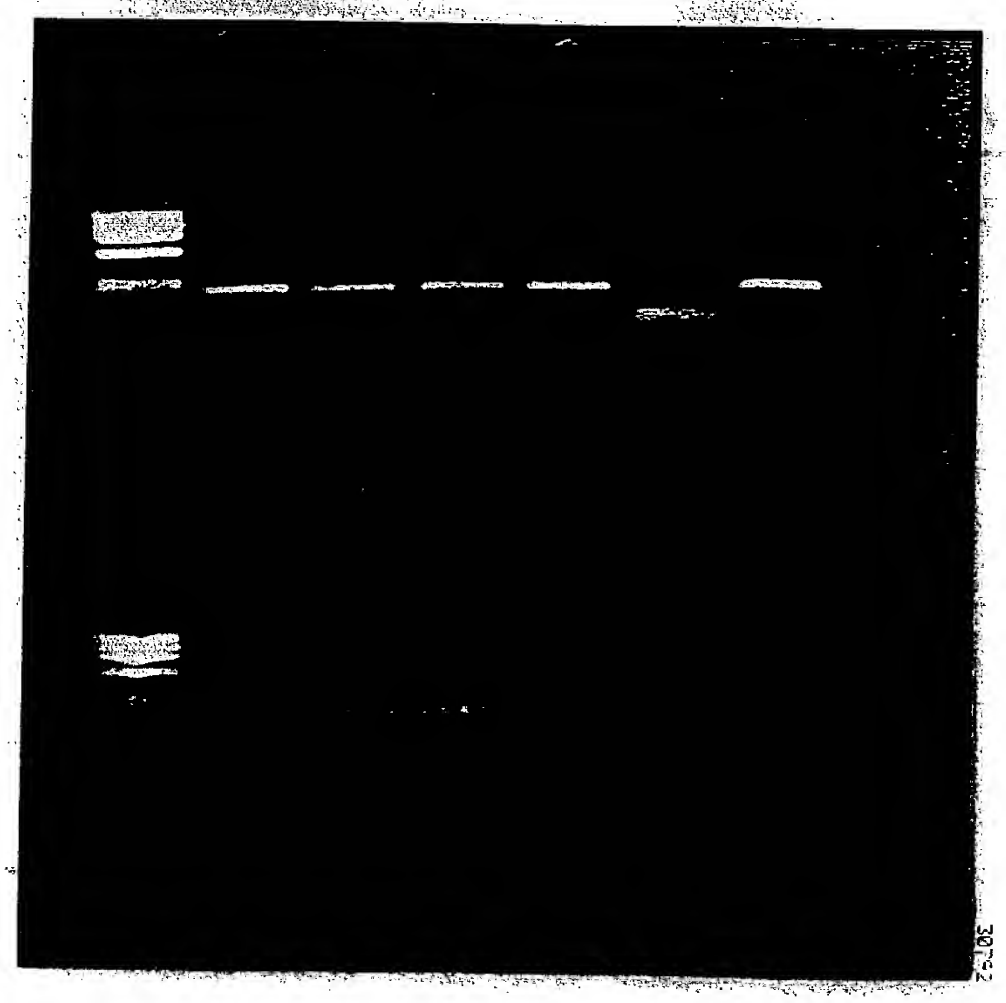
7/3/96

Do 2 10 + 1 H_2O 50 µl PCRs for new polymorphism-

REAGENT	VOLUME	
Buffer	55 µl	
$MgCl_2$	22 µl	
dNTP	44 µl	
Primer	~~55 µl~~	27 µl each
W-1	2.2	
Taq	2.2	
H_2O	330 µl	

4µl DNA per reac^n.

Contents of Sto 3:
step 0: temp: 96.0 deg C time: 0h 5m 0s slope: 2.00 deg/sec time-inc: 0 sec
begin cycle 35 times:
step 1: temp: 95.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 2: temp: 56.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 3: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
end cycle 35 times
step 4: temp: 72.0 deg C time: 0h 1m 0s slope: 2.00 deg/sec time-inc: 0 sec
step 5: temp: 4.0 deg C time:infinite slope: 2.00 deg/sec time-inc: 0 sec
total runtime (approx.): 2h49m21s

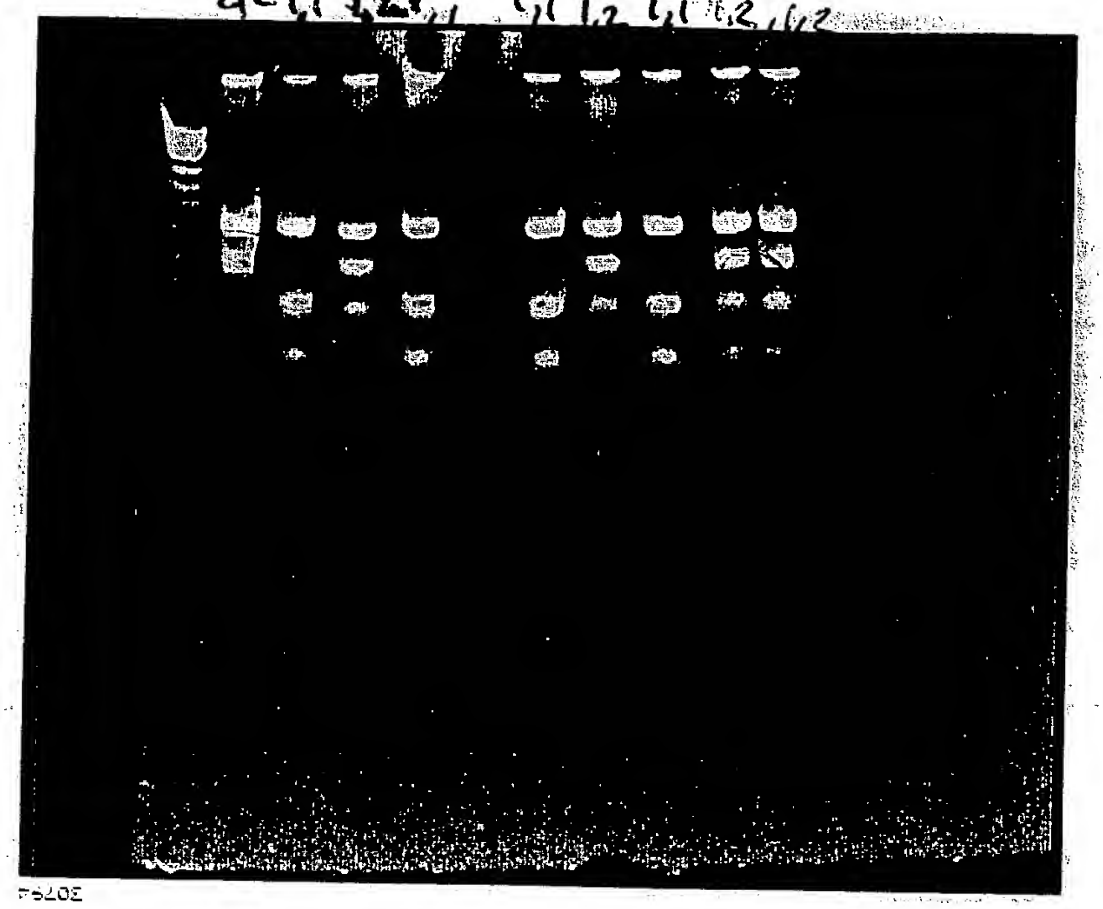


Set up digests o/n.

5 - 0.5µl enzyme / 10µl.
5 - 0.25µl enzyme / 10µl.

Reaction mix 1: 5µl enzyme + 15µl buffer
2: 2.5µl enzyme + 15µl buffer

leave to cleave @ 37°C overnight ⇒ Run on 9% polyacrylamide gel.



Unable to cleave the top band even with 10µl PCR product and 0.5µl enzyme @ 37°C overnight.-

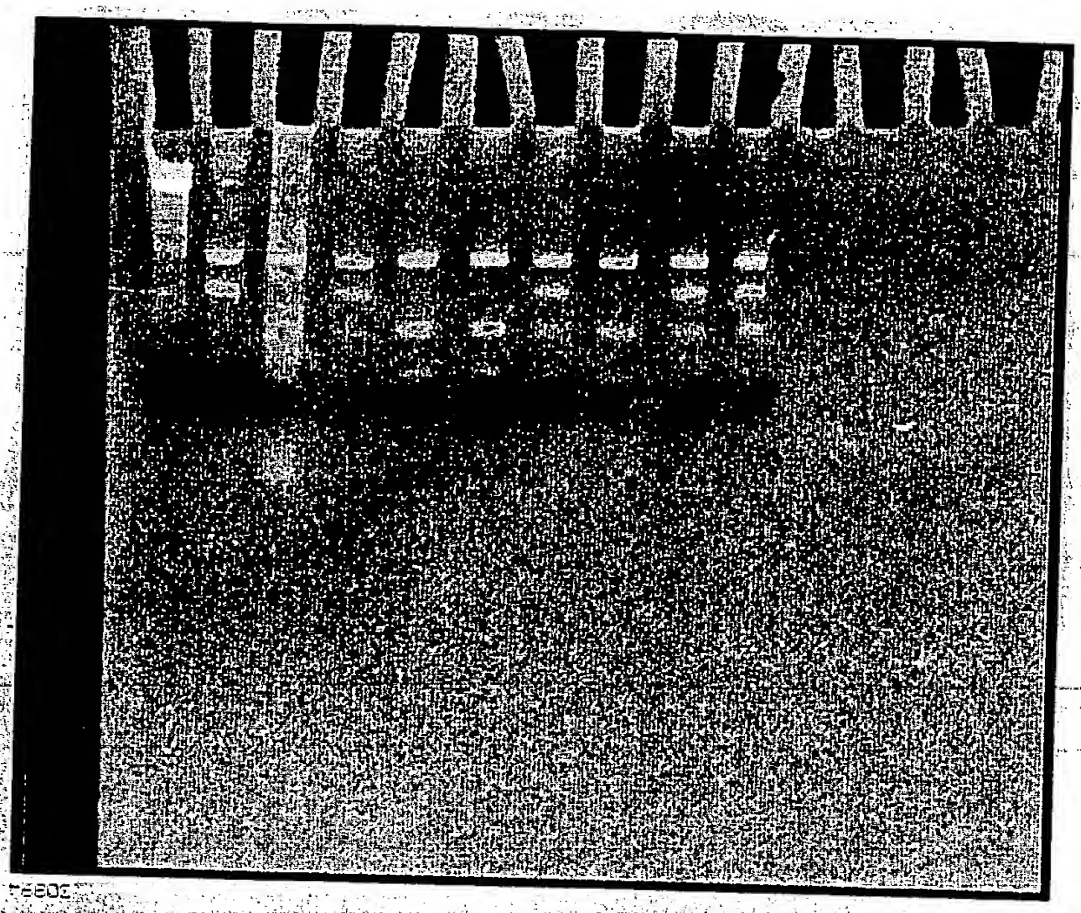
all samples run today were in equilibrium with +3953 polymorphism!

7	-	2,2	- GG
8	-	1,1	- CC
9	-	1,2	- GC
10	-	1,1	- CC
12	-	1,1	- CC
13	-	1,2	- GC
14	-	1,1	- CC
15	-	1,2	- GC
16	-	1,2	- GC

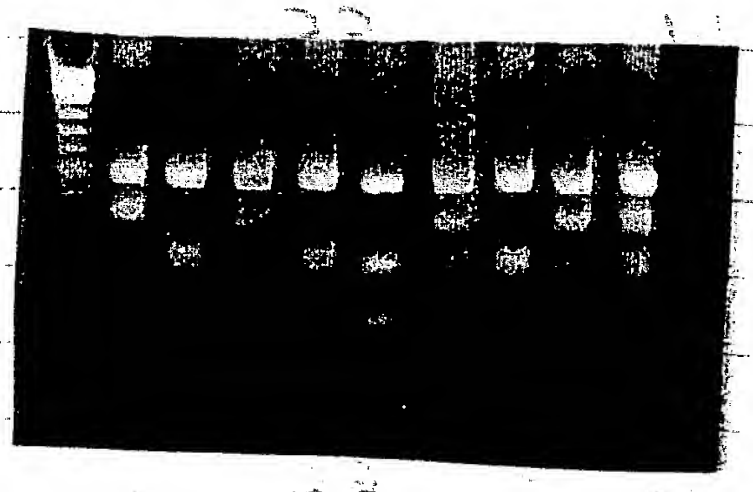
13/3

OPTIMISATION OF SCREENING FOR +6911

- dilute 7µl PCR product to 30µl with H_2O to cut out effects of buffers in PCR on enzyme.
- Add 3.5µl of buffer to each tube + 2µl enzyme. leave @ 37°C o/n.



Run ½ leave other ½ overnight for another night!



18/3/98

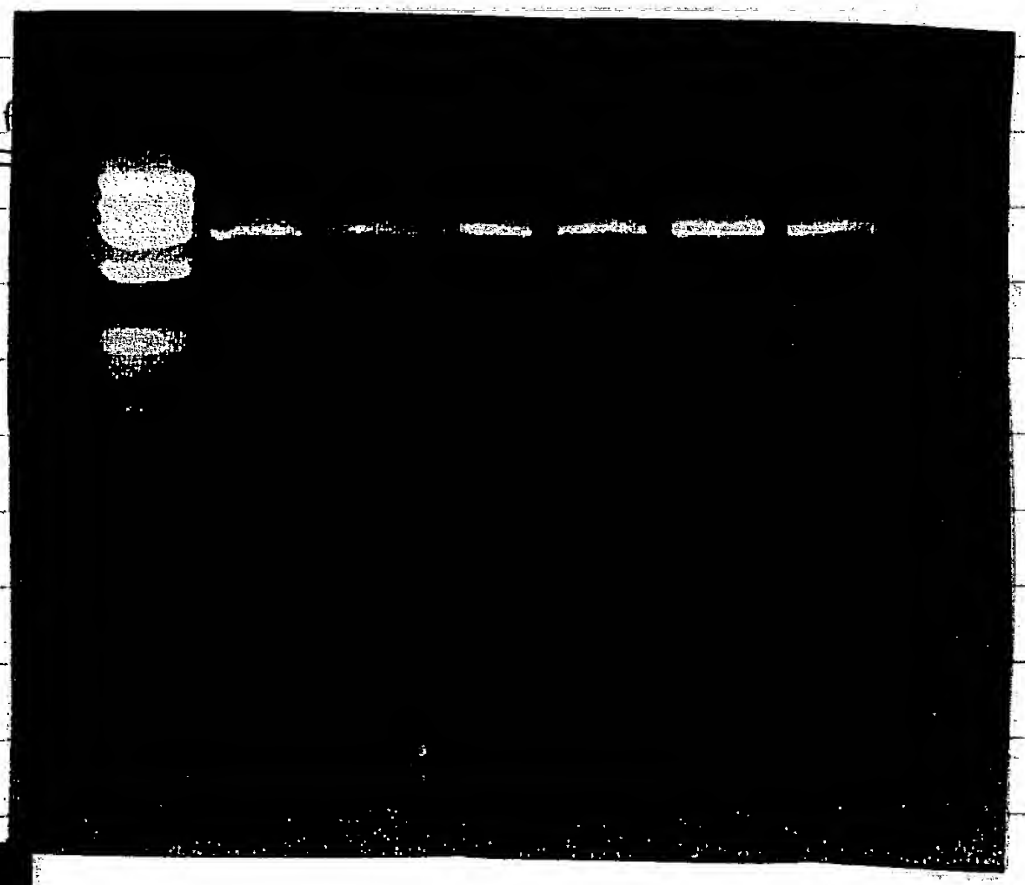
Do PCR's on 10 x F, B,

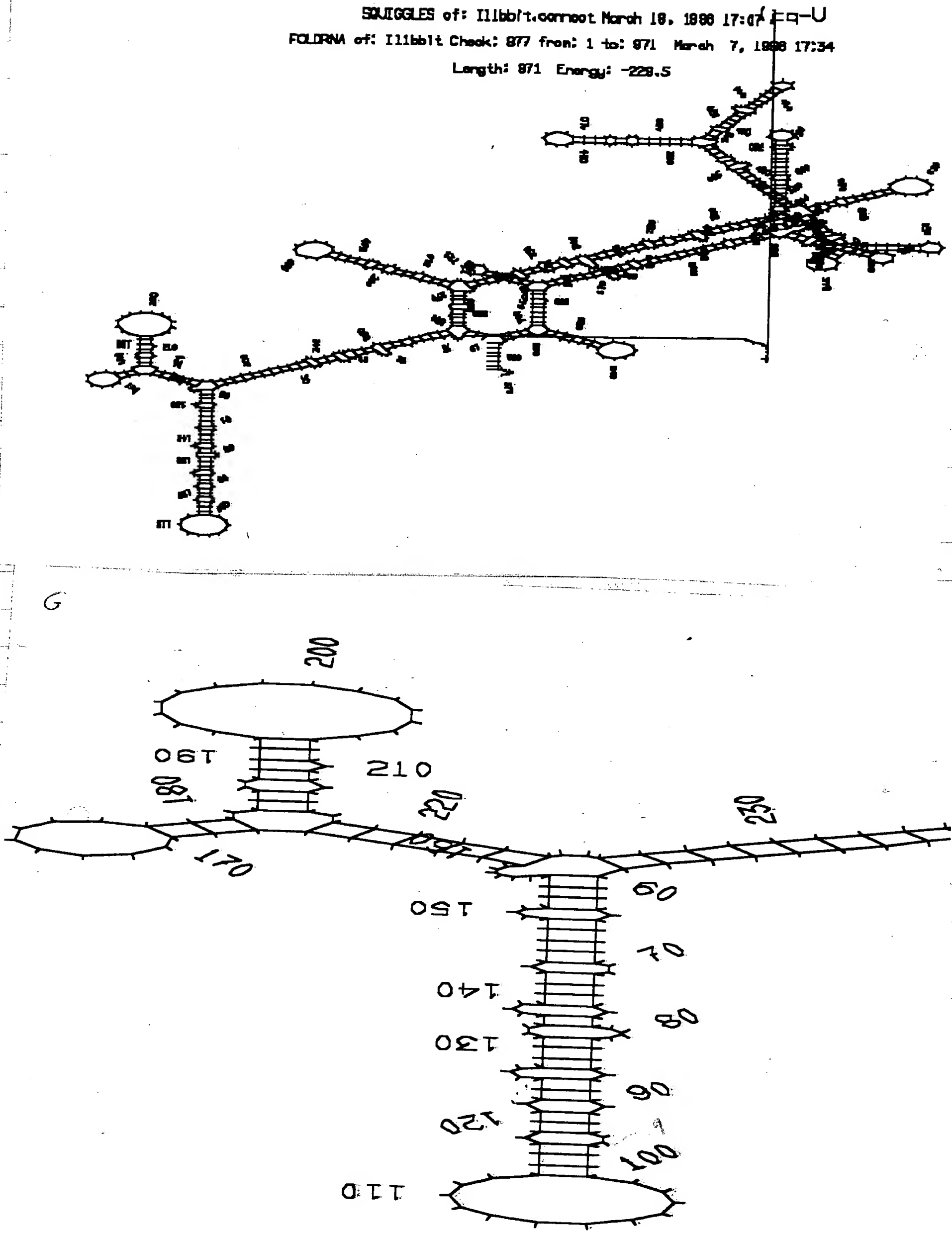
~~BUF~~ REAGENT	VOLUME
BUFFER	500
dNTP	400
$MgCl_2$	200
Primer	250 each - 100ul biotinylated
Taq	20
W1	20
H_2O	2960
	4600ul

Redo Taq

~~F/B~~ B_2

Running order
1) ~~ex haeIII~~
2) 153
3) 126
4) 114
5) 144
6) 139
7) 121

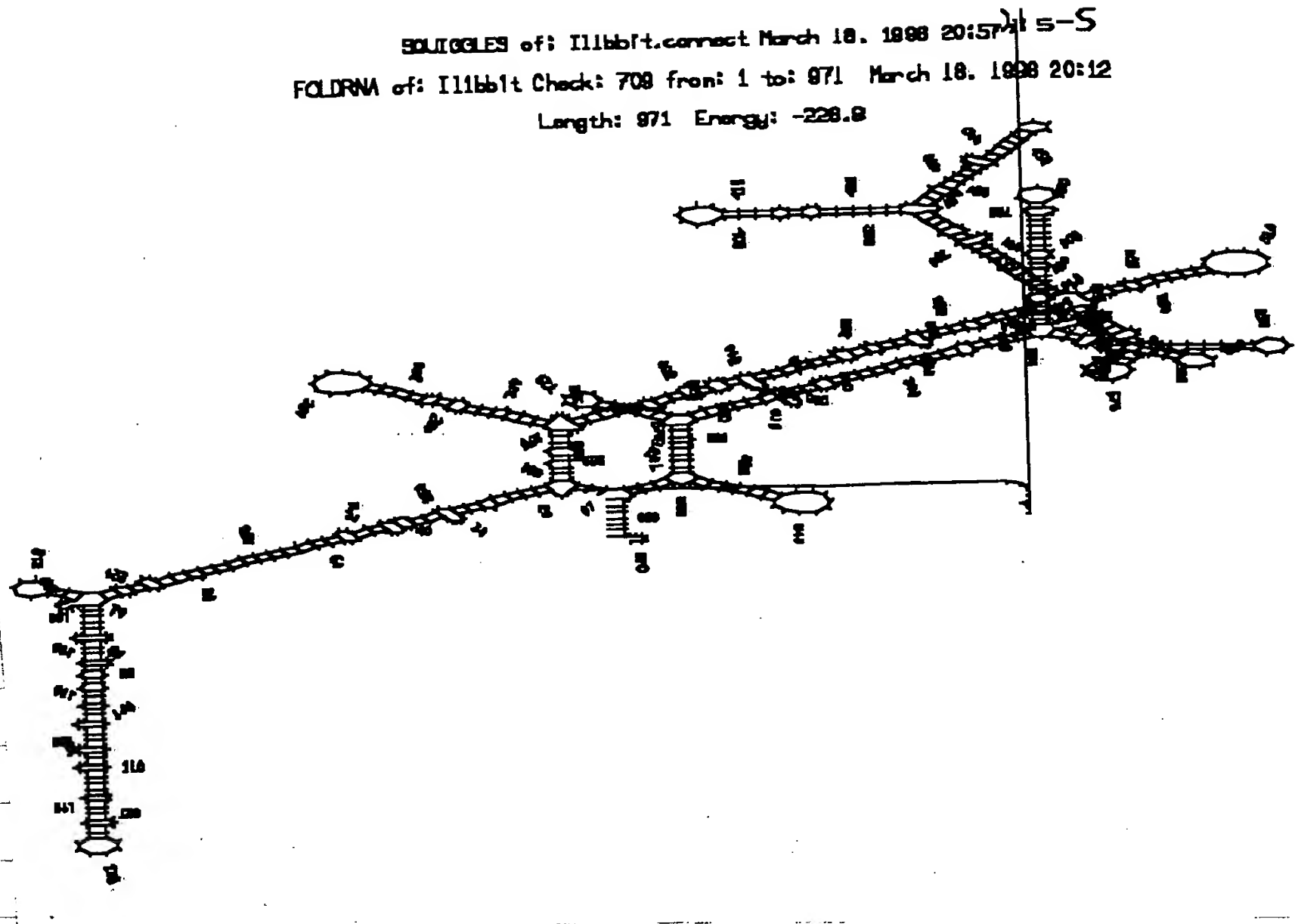


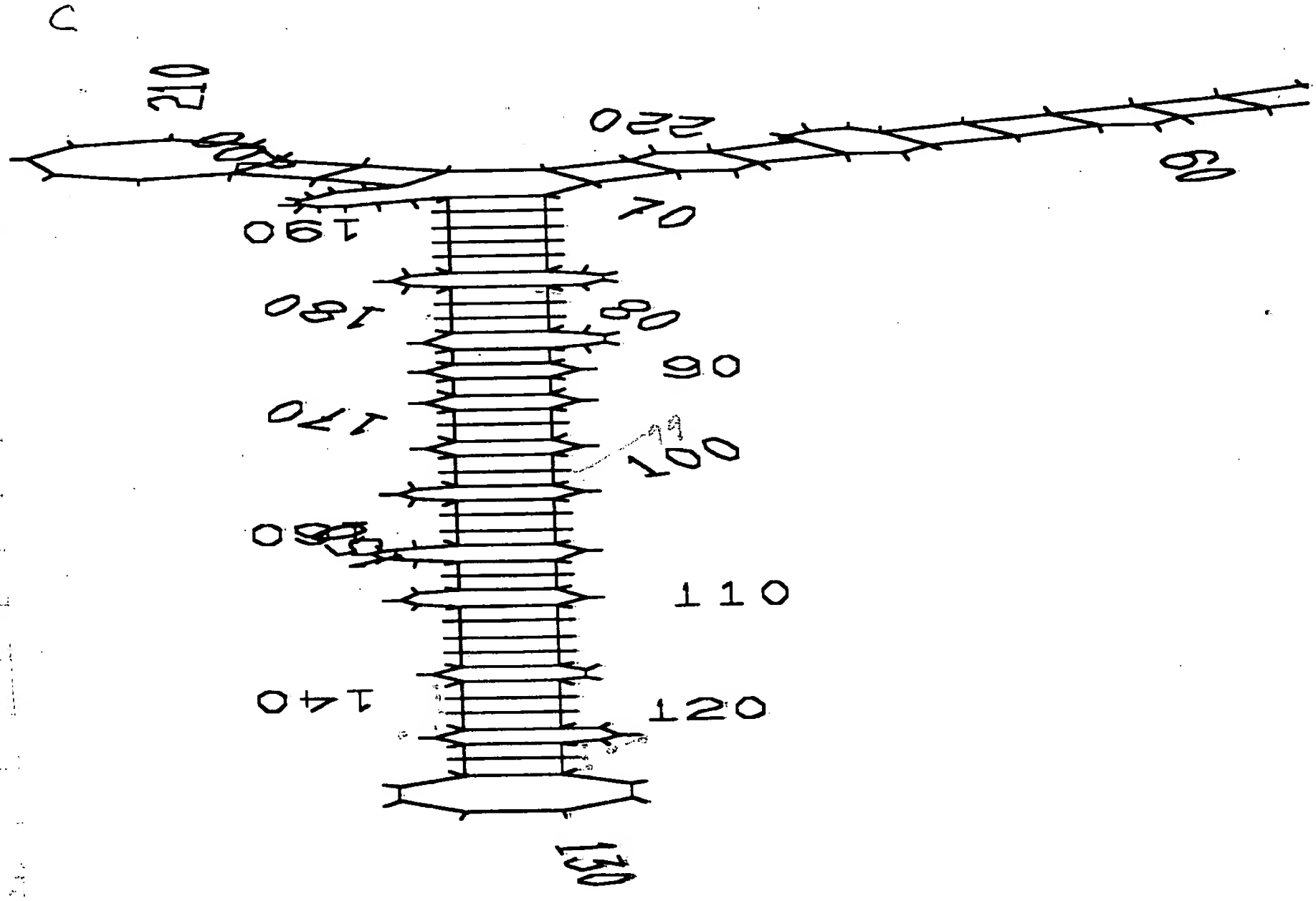

SQUIGGLES of: Illbbit.connect March 18, 1988 17:07
FOLDRNA of: Illbbit Check: 877 from: 1 to: 971 March 7, 1988 17:34
Length: 971 Energy: -228.5
200
190
210
180
220
230
170
60
150
70
140
80
130
90
120
100
110

G

MUTANT

136


SQUIGGLES of: I11bb1t.connect March 18. 1998 20:57
FOLDRNA of: I11bb1t Check: 708 from: 1 to: 971 March 18. 1998 20:12
Length: 971 Energy: -228.8
5-S
118
141


C
210
200
220
60
70
190
180
80
90
170
100
160
110
140
120
130

WT
151

22/3/96

Take Martins IL-1β probe in Puc 18 plasmid and plate out on Amp/met + IPTG + X Gal. Grow @ 37°C overnight - Take one white colony & inoculate 3 ml LB culture

Ampicillin

Meth

LB RECIPE 1 LITRE

10g Bacto trypton
5g Yeast extract
5g NaCl.

Autoclave before use.

3 ml culture was used to inoculate a large 1 litre culture (2 × 500 ml) Again ampicillin and methicillin were used.

Plasmid DNA is extracted by PEG precipitation to give good, pure yields. Using Ruth Herbsts Protocol.